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TITLE OF THE INVENTION (280 characters max)

Expression in Filamentous Fungi of Protease Inhibitors and Variants Thereof

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ENCLOSED APPLICATION PARTS (*check all that apply*)

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Respectfully submitted,

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Date: November 6, 2003 By: Carol A. See

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PROVISIONAL PATENT APPLICATION

EXPRESSION IN FILAMENTOUS FUNGI OF PROTEASE INHIBITORS AND VARIANTS THEREOF

NUMBER OF FIGURES: NINE (9) COMPRISING TWELVE (12) SHEETS

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EXPRESSION IN FILAMENTOUS FUNGI OF PROTEASE INHIBITORS AND VARIANTS THEREOF

FIELD OF THE INVENTION

[01] This invention relates to methods for the expression of protease inhibitors and variants thereof in filamentous fungi. The invention discloses fusion nucleic acids, vectors, fusion polypeptides, and processes for obtaining the protease inhibitors.

BACKGROUND OF THE INVENTION

[02] Proteases are involved in a wide variety of biological processes. Disruption of the balance between proteases and protease inhibitors is often associated with pathologic tissue destruction.

[03] Various studies have focused on the role of proteinases in tissue injury, and it is thought that the balance between proteinases and proteinase inhibitors is a major determinant in maintaining tissue integrity. Serine proteinases from inflammatory cells, including neutrophils, are implicated in various inflammatory disorders, such as pulmonary emphysema, arthritis, atopic dermatitis and psoriasis.

[04] Proteases also appear to function in the spread of certain cancers. Normal cells exist in contact with a complex protein network, called the extracellular matrix (ECM). The ECM is a barrier to cell movement and cancer cells must devise ways to break their attachments, degrade, and move through the ECM in order to metastasize. Proteases are enzymes that degrade other proteins and have long been thought to aid in freeing the tumor cells from their original location by chewing up the ECM. Recent studies have suggested that they may promote cell shape changes and motility through the activation of a protein in the tumor cell membrane called Protease-Activated Receptor-2 (PAR2). This leads to a cascade of intracellular reactions that activates the motility apparatus of the cell. Thus, it is hypothesized that one of the first steps in tumor metastasis is a reorganization of the cell shape such that it forms a distinct protrusion at one edge facing the direction of migration. The cell then migrates through a blood vessel wall travels to distal locations, eventually reattaching and forming a metastatic tumor. For example, human prostatic epithelial cells constitutively secrete prostate-specific antigen (PSA), a kallikrein-like serine protease, which is a normal component of the seminal plasma. The protease acts to degrade the extracellular matrix and facilitate invasion of cancerous cells.

[05] Synthetic and natural protease inhibitors have been shown to inhibit tumor promotion *in vivo* and *in vitro*. Previous research investigations have indicated that certain protease inhibitors belonging to a family of structurally-related proteins classified as serine protease inhibitors or SERPINS, are known to inhibit several proteases including trypsin, cathepsin G, thrombin, tissue kallikrein, as well as neutrophil elastase. The Serpins are extremely effective at preventing/suppressing carcinogen-induced transformation *in vitro* and carcinogenesis in animal model systems. Systemic delivery of purified protease inhibitors reduces joint inflammation and cartilage and bone destruction as well.

[06] Topical administration of protease inhibitors finds use in such conditions as atopic dermatitis, a common form of inflammation of the skin, which may be localized to a few patches or involve large portions of the body. The depigmenting activity of protease inhibitors and their capability to prevent ultraviolet-induced pigmentation have been demonstrated both *in vitro* and *in vivo*. Paine et al., *Journal of Investigative Dermatology* **116**, 587-595 (2001). Also, protease inhibitors have been found to help wound healing (http://www.sciencedaily.com/releases/2000/10/001002071718.htm). Secretory leukocyte protease inhibitor was demonstrated to reverse the tissue destruction and speed the wound healing process when applied topically. In addition, serine protease inhibitors can also help to reduce pain in lupus erythematosus patients (See US Patent No. 6537968).

[07] As noted above, protease inhibitors interfere with the action of proteases. Naturally occurring protease inhibitors can be found in a variety of foods such as cereal grains (oats, barley, and maize), Brussels sprouts, onion, beetroot, wheat, finger millet, and peanuts. One source of interest is the soybean. The average level in soybeans is around 1.4 percent and 0.6 percent for Kunitz and Bowman-Birk respectively, two of the most important protease inhibitors. These low levels make it impractical to isolate the natural protease inhibitor for clinical applications.

[08] Thus, there is a need for a method to produce large quantities of protease inhibitors and their variants that also reduces or eliminates the risk associated with blood-borne infectious agents when these agents are produced in mammalian tissue culture cells. The inventive production method provided for herein allows for the manufacture of large quantities of the protein therapeutic.

BRIEF SUMMARY OF THE INVENTION

[09] Provided herein are nucleic acids, cells and methods for the production of protease inhibitors and variants thereof.

[10] In a first embodiment, nucleic acids encoding a functional protease inhibitor are provided. In one aspect, a nucleic acid comprising regulatory sequences operatively linked to a first, second, third and fourth nucleic acid sequences are provided. Terminator sequences are provided following the fourth nucleic acid sequence.

[11] In a second aspect, the first nucleic acid sequence encodes a signal polypeptide functional as a secretory sequence in a first filamentous fungus, the second nucleic acid encodes a secreted polypeptide or functional portion thereof normally secreted from said first or a second filamentous fungus, the third nucleic acid encodes a cleavable linker and the fourth nucleic acid encodes a protease inhibitor or fragment thereof.

[12] In a third aspect, an expression cassette comprising nucleic acid sequences encoding a protease inhibitor is provided.

[13] In fourth aspect the present invention relates to a polynucleotide encoding a protease inhibitor variant. The polynucleotide may encode a Bowman-Birk Inhibitor variant wherein at least one loop has been altered. The polynucleotide may encode a Soybean Trypsin Inhibitor variant wherein at least one loop has been altered.

[14] In a second embodiment, methods of expressing a functional protease inhibitor or variant thereof are provided. In one aspect, a host cell is (i) transformed with an expression cassette comprising a nucleic acid sequence encoding a protease inhibitor or variant thereof, and (ii) cultured under appropriate conditions to express the protease inhibitor or variants thereof. Optionally, the method further comprises recovering the protease inhibitor or variant thereof.

[15] In a second aspect, a host cell is (i) transformed with an first expression cassette comprising a nucleic acid sequence encoding a protease inhibitor or variant thereof, (ii) transformed with a second expression cassette comprising a nucleic acid sequence encoding a chaperone, and (iii) cultured under appropriate conditions to express the protease inhibitors or variant thereof. Optionally, the protease inhibitors or variant thereof may be recovered. In one aspect, the protease inhibitors or variant thereof are expressed as a fusion protein. Optionally, the method further comprises recovering the protease inhibitor or variant thereof.

[16] In a third embodiment, cells capable of expressing a protease inhibitor or variant thereof is provided. Host cells are transformed an expression cassette encoding a protease inhibitor or variant thereof. Host cells may be selected from the group consisting of *Aspergillus* and *Trichoderma*.

[17] In a fourth embodiment, a functional protease inhibitor or variant thereof is provided. In one aspect, the functional protease inhibitor or variant thereof is expressed as a fusion protein consisting of the glucoamylase signal sequence, prosequence, catalytic domain and linker region up to amino acid number 502 of mature glucoamylase, followed by amino acids NVISKR and then by the mature protease inhibitor or variant thereof.

[18] In a second aspect, the expressed proteins are treated with a protease to liberate a protease inhibitor or variant thereof from the fusion protein.

[19] In a third aspect, the present invention provides a polypeptide having protease inhibitory activity, selected from the group consisting of

a) Bowman-Birk Inhibitor variants;

b) Soybean Trypsin Inhibitor variants;

c) Bowman-Birk Inhibitor;

d) Soybean Trypsin Inhibitor; and

e) A scaffold comprising at least one variant sequence.

[20] Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the scope and spirit of the invention will become apparent to one skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[21] Figure 1 is the codon optimized nucleotide sequence for soybean Bowman-Birk type protease inhibitor (BBI) (SEQ ID NO:1). This sequence includes nucleotides encoding NVISKR (dotted underline), the cleavage site for the fusion protein and three restriction enzyme sites for cloning into the expression plasmid. The *Nhe*I site at the 5' end and *Xho*I site at the 3' end are underlined and labeled. The *BstE*II site at the 3' end is designated by the # symbols. The stop codon is designated by the asterisks. There is no start codon as this is expressed as a fusion protein. The mature BBI coding sequence is indicated by the double underline (SEQ ID NO:2). The addition of nucleotides encoding three glycine (Figure 1B) residues prior to the mature BBI coding sequence can be done using the sequence encoding the three glycine residues indicated in Figure 2 (SEQ ID NO:5). Figure 1C nucleotide sequence encoding BBI, the three restriction sites, the kex2 site, three glycine residues at the N-terminal end and six histidine residues at the C-terminal end is shown (SEQ ID NO:76).

[22] Figure 2 is the codon optimized nucleotide sequence for Soybean Trypsin Inhibitor (STI), a Kunitz type protease inhibitor (SEQ ID NO:3). This sequence includes nucleotides encoding NVISKR (dotted underline) (SEQ ID NO:4), the cleavage site for the fusion protein, and six histidine residues at the C-terminal end (indicated by the dots). Three restriction enzyme sites (*Nhe*I at 5' end and *Xho*I and *BstE*II at 3' end, indicated as described for Figure 1) for cloning into the expression plasmid were also included. The three glycine residues after the kex2 site (NVISKR) are indicated by bold. The nucleotide sequence encoding the mature STI is indicated by the dashed underline (SEQ IN NO:6).

[23] Figure 3A is the mature amino acid sequence for BBI (SEQ ID NO:7). Figure 3B is BBI with three glycine residues at N-terminal (SEQ ID NO:8). Figure 3C is BBI with three glycine residues at N-terminal end and six histidine residues at C-terminal end (SEQ ID NO:9). In Figures 3A-C Loop1 is indicated by the underlined amino acid residues and Loop II amino acid residues are indicated by the bold type.

[24] Figure 4A is the mature amino acid sequence for STI (SEQ ID NO:10). Figure 4B is STI with three glycine residues at the N-terminal end (SEQ ID NO:11). Figure 4C is STI with three glycine residues at the N-terminus and with six histidine residues at the C-terminus (SEQ ID NO:12). Loop1 is indicated by the underlined amino acid residues (SEQ ID NO:13). Loop II amino acid residues are indicated by the bold type (SEQ ID NO:14).

[25] Figure 5 is a diagram of the expression plasmid pSLGAMpR2-BBI. This plasmid is based on pSLGAMpR2 which is derived from pSL1180 by inserting the *A. niger* glucoamylase promoter, catalytic core and terminator, a marker gene (*A. niger* pyrG) and a bovine prochymosin gene. The pSL1180 plasmid is available from Amersham Biosciences (Piscataway, NJ). The pSLGAMpR2 plasmid has the elements listed above inserted in the same relative location as shown for pSLGAMpR2-BBI except that the bovine prochymosin gene is located where the BBI gene. Thus, the BBI gene replaces the prochymosin gene in pSLGAMpR2 to yield pSLGAMpR2-BBI.

[26] Figure 6 is the amino acid sequences for wild-type BBI (SEQ ID NO:7) and select variants of BBI (SEQ ID NOs:15 thru 29). The wild-type BBI has the loops underlined. The differences in the variants from the wild-type are shown as either bold/underlined (Loop I) or bold (LoopII). In some variants, e.g., C2, C3, C4, C5 and Factor B, alanine at position13 (between two cysteines) was also changed to either "Serine", "Glycine" or "Glutamine". Also, compstatin peptide has 9 amino acids instead of 7. The variant sequences are also shown (SEQ ID NOs:30 thru 40).

[27] Figure 7 is a photograph of an agarose gel. Lane 1 contains molecular weight markers. Lane 2 is the untransformed parental strain. Lane 3 is the parental strain transformed with BBI-encoding DNA. Lane 4 is the parental strain co-transformed with a BBI-encoding vector and a chaperone (pdiA)-encoding vector. Lane 15 is the parental strain co-transformed with a BBI-encoding vector and a chaperone (prpA)-encoding vector. Expression of the desired protein, e.g., BBI, was enhanced in the presence of the chaperone.

[28] Figure 8 is a diagram of the plasmid pTrex4.

[29] Figure 9 A-D is the nucleic acid sequence for pTrex2 (SEQ ID NO:41).

DETAILED DESCRIPTION

[30] The invention will now be described in detail by way of reference only using the following definitions and examples. All patents and publications, including all sequences disclosed within such patents and publications, referred to herein are expressly incorporated by reference.

[31] Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Singleton, *et al.*, DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY, 2D ED., John Wiley and Sons, New York (1994), and Hale & Marham, THE HARPER COLLINS DICTIONARY OF BIOLOGY, Harper Perennial, NY (1991) provide one of skill with a general dictionary of many of the terms used in this invention. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described. Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively. Practitioners are particularly directed to Sambrook *et al.*, 1989, and Ausubel FM *et al.*, 1993, for definitions and terms of the art. It is to be understood that this invention is not limited to the particular methodology, protocols, and reagents described, as these may vary.

[32] The headings provided herein are not limitations of the various aspects or embodiments of the invention which can be had by reference to the specification as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to the specification as a whole.

DEFINITIONS

[33] An "expression cassette" or "expression vector" is a nucleic acid construct generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a particular nucleic acid in a target cell. The recombinant expression cassette can be incorporated into a plasmid, chromosome, mitochondrial DNA, plastid DNA, virus, or nucleic acid fragment. Typically, the recombinant expression cassette portion of an expression vector includes, among other sequences, a nucleic acid sequence to be transcribed and a promoter. Expression cassette may be used interchangeably with DNA construct and its grammatical equivalents.

[34] As used herein, the term "vector" refers to a nucleic acid construct designed for transfer nucleic acid sequences into cells. An "expression vector" refers to a vector that has the ability to incorporate and express heterologous DNA fragments in a foreign cell. Many prokaryotic and eukaryotic expression vectors are commercially available. Selection of appropriate expression vectors is within the knowledge of those having skill in the art.

[35] As used herein, the term "plasmid" refers to a circular double-stranded (ds) DNA construct used as a cloning vector, and which forms an extrachromosomal self-replicating genetic element in some eukaryotes or integrates into the host chromosomes.

[36] The term "nucleic acid molecule" or "nucleic acid sequence" includes RNA, DNA and cDNA molecules. It will be understood that, as a result of the degeneracy of the genetic code, a multitude of nucleotide sequences encoding a given protein may be produced.

[37] As used herein, a "fusion DNA sequence" comprises from 5' to 3' a first, second, third and fourth DNA sequences.

[38] As used herein, "a first nucleic acid sequence" or "first DNA sequence" encodes a signal peptide functional as a secretory sequence in a first filamentous fungus. Such signal sequences include those from glucoamylase, α-amylase and aspartyl proteases from *Aspergillus niger* var. *awamori, Aspergillus niger, Aspergillus oryzae*, signal sequences from cellobiohydrolase I, cellobiohydrolase II, endoglucanase I, endoglucanase III from *Trichoderma*, signal sequences from glucoamylase from *Neurospora* and *Humicola* as well as signal sequences from eukaryotes including the signal sequence from bovine chymosin, human tissue plasminogen activator, human interferon and synthetic consensus eukaryotic signal sequences such as that described by Gwynne *et al.* (1987) Bio/Technology 5, 713-719. Particularly preferred signal sequences are those derived from polypeptides secreted by the expression host used to express and secrete the fusion polypeptide. For example, the signal sequence from glucoamylase from *Aspergillus niger* is preferred when expressing

and secreting a fusion polypeptide from *Aspergillus niger*. As used herein, first amino acid sequences correspond to secretory sequences which are functional in a filamentous fungus. Such amino acid sequences are encoded by first DNA sequences as defined.

[39] As used herein, "second DNA sequences" encode "secreted polypeptides" normally expressed from filamentous fungi. Such secreted polypeptides include glucoamylase, α-amylase and aspartyl proteases from *Aspergillus niger* var. *awamori*, *Aspergillus niger*, and *Aspergillus oryzae*, cellobiohydrolase I, cellobiohydrolase II, endoglucanase I and endoglucanase III from *Trichoderma* and glucoamylase from *Neurospora* species and *Humicola* species. As with the first DNA sequences, preferred secreted polypeptides are those which are naturally secreted by the filamentous fungal expression host. Thus, for example when using *Aspergillus niger*, preferred secreted polypeptides are glucoamylase and α-amylase from *Aspergillus niger*, most preferably glucoamylase. In one aspect the glucoamylase is greater than 95%, 96%, 97%, 98% or 99% homologous with an *Aspergillus* glucoamylase.

[40] When *Aspergillus* glucoamylase is the secreted polypeptide encoded by the second DNA sequence, the whole protein or a portion thereof may be used, optionally including a prosequence. Thus, the cleavable linker polypeptide may be fused to glucoamylase at any amino acid residue from position 468 – 509. Other amino acid residues may be the fusion site but utilizing the above residues is particularly advantageous.

[41] A "functional portion of a secreted polypeptide" or grammatical equivalents means a truncated secreted polypeptide that retains its ability to fold into a normal, albeit truncated, configuration. For example, in the case of bovine chymosin production by *A. niger* var. *awamori* it has been shown that fusion of prochymosin following the 11th amino acid of mature glucoamylase provided no benefit compared to production of preprochymosin (US patent 5,364,770). In USSN 08/318,494, it was shown that fusion of prochymosin onto the C-terminus of preproglucoamylase up to the 297th amino acid of mature glucoamylase plus a repeat of amino acids 1-11 of mature glucoamylase yielded no secreted chymosin in *A. niger* var. *awamori*. In the latter case it is unlikely that the portion (approximately 63%) of the glucoamylase catalytic domain present in the fusion protein was able to fold correctly so that an aberrant, mis-folded and/or unstable fusion protein may have been produced which could not be secreted by the cell. The inability of the partial catalytic domain to fold correctly may have interfered with the folding of the attached chymosin. Thus, it is likely that sufficient residues of a domain of the naturally secreted polypeptide must be present to allow it to fold in its normal configuration independently of the desired polypeptide to which it is attached.

[42] In most cases, the portion of the secreted polypeptide will be both correctly folded and result in increased secretion as compared to its absence.

[43] Similarly, in most cases, the truncation of the secreted polypeptide means that the functional portion retains a biological function. In a preferred embodiment, the catalytic domain of a secreted polypeptide is used, although other functional domains may be used, for example, the substrate binding domains. In the case of *Aspergillus niger* and *Aspergillus niger* var. *awamori* glucoamylase, preferred functional portions retain the catalytic domain of the enzyme, and include amino acids 1-471. Additionally preferred embodiments utilize the catalytic domain and all or part of the linker region. Alternatively, the starch binding domain of glucoamylase may be used, which comprises amino acids 509-616 of *Aspergillus niger* and *Aspergillus niger* var. *awamori* glucoamylase.

[44] As used herein, "third DNA sequences" comprise DNA sequences encoding a cleavable linker polypeptide. Such sequences include those which encode the prosequence of glucoamylase, the prosequence of bovine chymosin, the prosequence of subtilisin, prosequences of retroviral proteases including human immunodeficiency virus protease and DNA sequences encoding amino acid sequences recognized and cleaved by trypsin, factor X_a collagenase, clostripin, subtilisin, chymosin, yeast KEX2 protease, *Aspergillus* KEXB and the like. See e.g. Marston, F.A.O. (1986) Biol. Chem J. 240, 1-12. Such third DNA sequences may also encode the amino acid methionine that may be selectively cleaved by cyanogen bromide. It should be understood that the third DNA sequence need only encode that amino acid sequence which is necessary to be recognized by a particular enzyme or chemical agent to bring about cleavage of the fusion polypeptide. Thus, the entire prosequence of, for example, glucoamylase, chymosin or subtilisin need not be used. Rather, only that portion of the prosequence which is necessary for recognition and cleavage by the appropriate enzyme is required.

[45] It should be understood that the third nucleic acid need only encode that amino acid sequence which is necessary to be recognized by a particular enzyme or chemical agent to bring about cleavage of the fusion polypeptide.

[46] Particularly preferred cleavable linkers are the KEX2 protease recognition site (Lys-Arg), which can be cleaved by a native *Aspergillus* KEX2-like (KEXB) protease, trypsin protease recognition sites of Lys and Arg, and the cleavage recognition site for endoproteinase-Lys-C.

[47] As used herein, "fourth DNA sequences" encode "desired polypeptides." Such desired polypeptides include protease inhibitors and variants thereof.

[48] The above-defined four DNA sequences encoding the corresponding four amino acid sequences are combined to form a "fusion DNA sequence." Such fusion DNA sequences are assembled in proper reading frame from the 5' terminus to 3' terminus in the order of first, second, third and fourth DNA sequences. As so assembled, the DNA sequence will encode a "fusion polypeptide" or "fusion protein" or "fusion analog" encoding from its amino-terminus a signal peptide functional as a secretory sequence in a filamentous fungus, a secreted polypeptide or portion thereof normally secreted from a filamentous fungus, a cleavable linker polypeptide and a desired polypeptide.

[49] As used herein, the terms "desired protein" or "desired polypeptide" refers to a polypeptide or protein in its mature form that is not fused to a secretion enhancing construct. Thus, a "desired protein" or "desired polypeptide" refers to the protein to be expressed and secreted by the host cell in a non-fused form.

[50] As used herein, a "fusion polypeptide" or "fusion protein" or "fusion analog" encodes from its amino-terminus a signal peptide functional as a secretory sequence functional in a host cell, a secreted polypeptide or portion thereof normally secreted from a host cell, a cleavable linker polypeptide and a desired polypeptide. The fusion protein may be processed by host cell enzymes, e.g., a protease, to yield the desired protein free from the other protein sequences in the fusion protein. As used herein, the terms "fusion analog" or "fusion polypeptide" or "fusion protein" may be used interchangeably.

[51] As used herein, a "promotor sequence" is a DNA sequence which is recognized by the particular filamentous fungus for expression purposes. It is operably linked to a DNA sequence encoding the above defined fusion polypeptide. Such linkage comprises positioning of the promoter with respect to the translation initiation codon of the DNA sequence encoding the fusion DNA sequence. The promoter sequence contains transcription and translation control sequences which mediate the expression of the fusion DNA sequence. Examples include the promoter from the *A. niger* var. *awamori* or *A. niger* glucoamylase genes (Nunberg, J.H. et al. (1984) Mol. Cell. Biol. 4, 2306-2315; Boel, E. et al. (1984) EMB0 J. 3, 1581-1585), the *A. oryzae, A. niger* var. *awamori* or *A. niger* or alpha-amylase genes, the *Rhizomucor miehei* carboxyl protease gene, the *Trichoderma reesei* cellobiohydrolase I gene (Shoemaker, S.P. et al. (1984) European Patent Application No. EPO0137280A1), the *A. nidulans trpC* gene (Yelton, M. et al. (1984) Proc. Natl. Acad. Sci. USA 81, 1470-1474; Mullaney, E.J. et al. (1985) Mol. Gen. Genet. 199, 37-45) the *A. nidulans alcA* gene (Lockington, R.A. et al. (1986) Gene 33 137-149), the *A. nidulans amdS* gene (McKnight, G.L. et al. (1986) Cell 46, 143-147), the *A. nidulans amdS* gene (Hynes,

M.J. et al. (1983) Mol. Cell Biol. 3, 1430-1439), and higher eukaryotic promoters such as the SV40 early promoter (Barclay, S.L. and E. Meller (1983) Molecular and Cellular Biology 3, 2117-2130).

[52] Likewise a "terminator sequence" is a DNA sequence which is recognized by the expression host to terminate transcription. It is operably linked to the 3' end of the fusion DNA encoding the fusion polypeptide to be expressed. Examples include the terminator from the *A. nidulans trpC* gene (Yelton, M. et al. (1984) Proc. Natl. Acad. Sci. USA 81, 1470-1474; Mullaney, E.J. et al. (1985) Mol. Gen. Genet. 199, 37-45), the *A. niger* var. *awamori* or *A. niger* glucoamylase genes (Nunberg, J.H. et al. (1984) Mol. Cell. Biol. 4, 2306-253; Boel, E. et al. (1984) EMBO J. 3, 1581-1585), the *A. oryzae, A. niger* var. *awamori* or *A. niger* or alpha-amylase genes and the *Rhizomucor miehei* carboxyl protease gene (EPO Publication No. 0 215 594), although any fungal terminator is likely to be functional in the present invention.

[53] A "polyadenylation sequence" is a DNA sequence which when transcribed is recognized by the expression host to add polyadenosine residues to transcribed mRNA. It is operably linked to the 3' end of the fusion DNA encoding the fusion polypeptide to be expressed. Examples include polyadenylation sequences from the *A. nidulans trpC* gene (Yelton, M. et al. (1984) Proc. Natl. Acad. Sci. USA 81, 1470-1474; Mullaney, E.J. et al. (1985) Mol. Gen. Genet. 199, 37-45), the *A. niger* var. *awamori* or *A. niger* glucoamylase genes (Nunberg, J.H. et al. (1984) Mol. Cell. Biol. 4, 2306-2315) (Boel, E. et al. (1984) EMBO J. 3, 1581-1585), the *A. oryzae, A. niger* var. *awamori* or *A. niger* or alpha-amylase genes and the *Rhizomucor miehei* carboxyl protease gene described above. Any fungal polyadenylation sequence, however, is likely to be functional in the present invention.

[54] As used herein, the term "selectable marker-encoding nucleotide sequence" refers to a nucleotide sequence which is capable of expression in fungal cells and where expression of the selectable marker confers to cells containing the expressed gene the ability to grow in the presence of a corresponding selective condition.

[55] A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA encoding a secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a

secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

[56] As used herein, "recombinant" includes reference to a cell or vector, that has been modified by the introduction of a heterologous nucleic acid sequence or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found in identical form within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all as a result of deliberate human intervention.

[57] As used herein, the term "expression" refers to the process by which a polypeptide is produced based on the nucleic acid sequence of a gene. The process includes both transcription and translation. It follows that the term "protease inhibitor expression" refers to transcription and translation of the specific protease inhibitors and variants thereof gene to be expressed, the products of which include precursor RNA, mRNA, polypeptide, post-translation processed polypeptide, and derivatives thereof. Similarly, "protease inhibitor expression" refers to the transcription, translation and assembly of protease inhibitors and variants thereof into a form exemplified by Figure 6. By way of example, assays for protease inhibitor expression include examination of fungal colonies when exposed to the appropriate conditions, western blot for protease inhibitor protein, as well as northern blot analysis and reverse transcriptase polymerase chain reaction (RT-PCR) assays for protease inhibitor mRNA.

[58] As used herein the term "glycosylated" means that oligosaccharide molecules have been added to particular amino acid residues on a protein. A "de-glycosylated" protein is a protein that has been treated to partially or completely remove the oligosaccharide molecules from the protein. An "aglycosylated" protein is a protein that has not had the oligosaccharide molecules added to the protein. This may be due to a mutation in the protein that prevents the addition of the oligosaccharide.

[59] A "non-glycosylated" protein is a protein that does not have the oligosaccharide attached to the protein. This may be due to various reasons, including but not limited to, the absence of enzymes responsible for the addition of the oligosaccharides to proteins. The term "non-glycosylated" encompasses both proteins that have not had the oligosaccharide added to the protein and those in which the oligosaccharides have been added but were subsequently removed. An "aglycosylated" protein may be a "non-glycosylated" protein. A

"non-glycosylated" protein may be either an "aglycosylated" protein or a "deglycosylated" protein.

[60] The terms "isolated" or "purified" as used herein refer to a nucleic acid or polypeptide that is removed from at least one component with which it is naturally associated

[61] The term "substantially free" includes preparations of the desired polypeptide having less than about 20% (by dry weight) other proteins (i.e., contaminating protein), less than about 10% other proteins, less than about 5% other proteins, or less than about 1% other proteins.

[62] The term "substantially pure" when applied to the proteins or fragments thereof of the present invention means that the proteins are essentially free of other substances to an extent practical and appropriate for their intended use. In particular, the proteins are sufficiently pure and are sufficiently free from other biological constituents of the host cells so as to be useful in, for example, protein sequencing, or producing pharmaceutical preparations.

[63] The term "target protein" as used herein refers to protein, e.g., an enzyme, hormone or the like, whose action would be blocked by the binding of the variant inhibitors provided for herein.

[64] The terms "variant sequence" or "variant sequences" refer to the short polypeptide sequence(s) that replace the binding loops of the wild-type protease inhibitor or other scaffold. The variant sequence does not need to be of the same length as the binding loop sequence it is replacing in the scaffold.

[65] The term "scaffold" refers to the wild-type protein sequence into which a variant sequence may be introduced. In an embodiment the scaffold will have portions, e.g., loops, that may be replaced. For example, the STI and BBI sequences used herein would be a scaffold for a variant sequence.

PROTEASE INHIBITORS

[66] Two protein protease inhibitors have been isolated from soybeans, the Kunitz-type trypsin inhibitor (soybean trypsin inhibitor, STI) and the Bowman-Birk protease inhibitor (BBI). See, e.g., Birk, Int. J. Pept. Protein Res. 25:113-131 (1985) and Kennedy, Am. J. Clin. Neutr. 68:1406S-1412S (1998). These inhibitors serve as a scaffold for the variant sequences.

[67] In addition, to alterations in the scaffold comprising the variant sequences, other desired proteins used herein include the addition of three glycine residues at the N-terminal and/or six histidine residues at the C-terminal. See Figures 3 and 4.

Soybean Trypsin Inhibitor (STI)

[68] STI inhibits the proteolytic activity of trypsin by the formation of a stable stoichiometric complex. See, e.g., Liu, K., Chemistry and Nutritional value of soybean components. In: Soybeans, chemistry, technology and utilization. pp. 32-35 (Aspen publishers, Inc., Gaithersburg, Md., 1999). STI consists of 181 amino acid residues with two disulfide bridges and is roughly spherically shaped. See, e.g., Song et al., J. Mol. Biol. 275:347-63 (1998). The two disulfide bridges form two binding loops similar to those described below for BBI.

[69] The Kunitz-type soybean trypsin inhibitor (STI) has played a key role in the early study of proteinases, having been used as the main substrate in the biochemical and kinetic work that led to the definition of the standard mechanism of action of proteinase inhibitors.

Bowman-Birk Inhibitor (BBI)

[70] BBI proteins are a kinetically and structurally well-characterized family of small proteins (60-90 residues) isolated from leguminous seeds. They have a symmetrical structure of two tricyclic domains each containing an independent binding loop. Loop I typically inhibits trypsin and loop II chymotrypsin (Chen et al., J. Biol. Chem. (1992) 267:1990-1994; Werner & Wemmer, 1992; Lin et al., Eur. J. Biochem. (1993) 212:549-555; Voss et al., Eur. J. Biochem. (1996) 242:122-131). These binding regions each contain a ``canonical loop" structure, which is a motif found in a variety of serine proteinase inhibitors (Bode & Huber, Eur. J. Biochem. (1992) 204:433-451).

[71] BBI is an 8 k-Da protein that inhibits the proteases trypsin and chymotrypsin at separate reactive sites. See, e.g., Billings et al., Pro. Natl. Acad. Sci. 89:3120-3124 (1992). STI and BBI are found only in the soybean seed, and not in any other part of the plant. See, e.g., Birk, Int. J. Pept. Protein Res. 25:113-131 (1985).

[72] Although numerous isoforms of BBI have been characterized, SEQ ID NO: 7 (Figure 3) shows the amino acid sequence of the BBI backbone used herein comprising approximately 71 amino acid residues. In addition, BBI may become truncated with as many as 10 amino acid residues being removed from either the N- or C- terminal. For example, upon seed desiccation, a BBI many have the C-terminal 9 or 10 amino acid residues removed. Thus, proteolysis is highly tolerated prior to the initial disulphide and just after the terminal disulphide bond, the consequences of which are usually not detrimental to the

binding to target protein. However, it will be appreciated that any one of the isoforms or truncated forms could be used.

Protease Inhibitor Variants

[73] As noted above, the STI and BBI protease inhibitors have binding loops that inhibit proteases. The inventive protease inhibitor variants provided for herein have alterations in Loop I, Loop II or both loops. In an embodiment, the loops are replaced with sequences that interact with a target protein.

[74] The loops can be replaced with sequences derived from VEGF binding proteins, inhibitors of the complement pathway such as C2, C3, C4 or C5 inhibitors, cotton binding proteins, Compstatin and the like. Alternatively, variant sequences can be selected by various methods known in the art such as, for example, phage display or other screening method. For example, a random peptide gene library is fused with phage PIII gene so the peptide library will be displayed on the surface of the phage. Subsequently, the phage display library is exposed to the target protein and washed with buffer to remove non-specific binding (this process is sometimes referred to as panning). Finally, the binding phage and PCR the DNA sequence for the peptide encoded are isolated.

[75] Generally, a loop will be replaced with a variant sequence, i.e., peptides, 3 to 14 amino acids in length, 5 to 10 amino acids being preferred. Longer sequences may be used as long as they provide the binding and/or inhibition desired. In addition, peptides suitable for use as replacements of the binding loop(s) should adopt a functional conformation when contained within a constrained loop, i.e., a loop formed by the presence to a disulfide bond between two cysteine residues. In specific embodiments, the peptides are between 7 and 9 amino acids in length. These replacement sequences also provide protease inhibition or binding to the targeted proteins.

[76] In some cases it may be advantages to alter a single amino acid. Specifically, the Alanine at residue 13 of wild-type STI or BBI may be changed to a Serine, a Glycine or a Glutamine.

FUSION PROTEINS

[77] Each protease inhibitor and variant thereof will be expressed as a fusion protein by the host fungal cell. Although cleavage of the fusion polypeptide to release the desired protein will often be useful, it is not necessary. Protease inhibitors and variants thereof expressed and secreted as fusion proteins surprisingly retain their function.

[78] The above-defined four DNA sequences encoding the corresponding four amino acid sequences are combined to form a "fusion DNA sequence." Such fusion DNA sequences

are assembled in proper reading frame from the 5' terminus to 3' terminus in the order of first, second, third and fourth DNA sequences. As so assembled, the DNA sequence will encode a "fusion polypeptide" encoding from its amino-terminus a signal peptide functional as a secretory sequence in a filamentous fungus, a secreted polypeptide or portion thereof normally secreted from a filamentous fungus, a cleavable linker peptide and a desired polypeptide, e.g., a protease inhibitor and variants thereof.

[79] Production of fusion proteins can be accomplished by use of the methods disclosed in, for example, US Patents 5,411,873, 5,429,950, and 5,679,543. Other methods are well known in the art.

EXPRESSION OF RECOMBINANT A PROTEASE INHIBITOR

[80] To the extent that this invention depends on the production of fusion proteins, it relies on routine techniques in the field of recombinant genetics. Basic texts disclosing the general methods of use in this invention include Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual* (2nd ed. 1989); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and Ausubel *et al.*, eds., *Current Protocols in Molecular Biology* (1994).

[81] This invention provides filamentous fungal host cells which have been transduced, transformed or transfected with an expression vector comprising a protease inhibitor-encoding nucleic acid sequence. The culture conditions, such as temperature, pH and the like, are those previously used for the parental host cell prior to transduction, transformation or transfection and will be apparent to those skilled in the art.

[82] Basically, a nucleotide sequence encoding a fusion protein is operably linked to a promoter sequence functional in the host cell. This promoter-gene unit is then typically cloned into intermediate vectors before transformation into the host cells for replication and/or expression. These intermediate vectors are typically prokaryotic vectors, e.g., plasmids, or shuttle vectors.

[83] In one approach, a filamentous fungal cell line is transfected with an expression vector having a promoter or biologically active promoter fragment or one or more (e.g., a series) of enhancers which functions in the host cell line, operably linked to a nucleic acid sequence encoding a protease inhibitor, such that the a protease is expressed in the cell line. In a preferred embodiment, the DNA sequences encode a protease inhibitor or variant thereof. In another preferred embodiment, the promoter is a regulatable one.

A. Condon Optimization

[84] Optimizing codon usage in genes that express well with those genes that do not express well is known in the art. See Barnett et al., GB2200118 and Bergquist et al., Extremophiles (2002) 6:177-184. Codon optimization, as used herein, was based on comparing heterologous proteins that are expressed well in *Aspergillus* and native secreted proteins to the heterlogous proteins that are not expressed well. See Table I.

Table I:

Proteins that expressed well	**Proteins that did not express well**
glucoamylase alpha-amylase stachybotrys laccase A stachybotrys laccase B human trypsin SCCE bovine prochymosin Her2 antibodies light chain	Human DPPIV NEP

[85] Selected codons that were not used or not used often in the expressed proteins will be changed to codons that were used often. Therefore, we only changed a subset of codons.

B. Nucleic Acid Constructs/Expression Vectors.

[86] Natural or synthetic polynucleotide fragments encoding a protease inhibitor ("PI-encoding nucleic acid sequences") may be incorporated into heterologous nucleic acid constructs or vectors, capable of introduction into, and replication in, a filamentous fungal cell. The vectors and methods disclosed herein are suitable for use in host cells for the expression of a protease inhibitor and variants thereof. Any vector may be used as long as it is replicable and viable in the cells into which it is introduced. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. Appropriate cloning and expression vectors for use in filamentous fungal cells are also described in Sambrook *et al.*, 1989, and Ausubel FM *et al.*, 1989, expressly incorporated by reference herein. The appropriate DNA sequence may be inserted into a plasmid or vector (collectively referred to herein as "vectors") by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by standard procedures. Such procedures and related sub-cloning procedures are deemed to be within the scope of knowledge of those skilled in the art.

[87] Appropriate vectors are typically equipped with a selectable marker-encoding nucleic acid sequence, insertion sites, and suitable control elements, such as termination sequences. The vector may comprise regulatory sequences, including, for example, non-coding sequences, such as introns and control elements, *i.e.*, promoter and terminator elements or 5' and/or 3' untranslated regions, effective for expression of the coding sequence in host cells (and/or in a vector or host cell environment in which a modified soluble protein coding sequence is not normally expressed), operably linked to the coding sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, many of which are commercially available and/or are described in Sambrook, *et al.*, (*supra*).

[88] Exemplary promoters include both constitutive promoters and inducible promoters, examples of which include a CMV promoter, an SV40 early promoter, an RSV promoter, an EF-1α promoter, a promoter containing the tet responsive element (TRE) in the tet-on or tet-off system as described (ClonTech and BASF), the beta actin promoter and the metallothionein promoter that can upregulated by addition of certain metal salts. In one embodiment of this invention, *glaA* promoter is used. This promoter is induced in the presence of maltose. Such promoters are well known to those of skill in the art.

[89] Those skilled in the art are aware that a natural promoter can be modified by replacement, substitution, addition or elimination of one or more nucleotides without changing its function. The practice of the invention encompasses and is not constrained by such alterations to the promoter.

[90] The choice of promoter used in the genetic construct is within the knowledge of one skilled in the art.

[91] The choice of the proper selectable marker will depend on the host cell, and appropriate markers for different hosts are well known in the art. Typical selectable marker genes encode proteins that (a) confer resistance to antibiotics or other toxins, *for example*, ampicillin, methotrexate, tetracycline, neomycin (Southern and Berg, J., 1982), mycophenolic acid (Mulligan and Berg, 1980), puromycin, zeomycin, or hygromycin (Sugden et al., 1985) or (b) compliment an auxotrophic mutation or a naturally occurring nutritional deficiency in the host strain. In a preferred embodiment, a fungal *pyrG* gene is used as a selectable marker (Ballance, D.J. et al., 1983, Biochem. Biophys. Res. Commun. 112:284-289). In another preferred embodiment, a fungal *amdS* gene is used as a selectable marker (Tilburn, J. et al., 1983, Gene 26:205-221).

[92] A selected PI coding sequence may be inserted into a suitable vector according to well-known recombinant techniques and used to transform a cell line capable of PI expression. Due to the inherent degeneracy of the genetic code, other nucleic acid sequences which encode substantially the same or a functionally equivalent amino acid sequence may be used to clone and express a specific protease inhibitor, as further detailed above. Therefore it is appreciated that such substitutions in the coding region fall within the sequence variants covered by the present invention. Any and all of these sequence variants can be utilized in the same way as described herein for a parent PI-encoding nucleic acid sequence. One skilled in the art will recognize that differing PIs will be encoded by differing nucleic acid sequences.

[93] Once the desired form of a protease inhibitor nucleic acid sequence, homologue, variant or fragment thereof, is obtained, it may be modified in a variety of ways. Where the sequence involves non-coding flanking regions, the flanking regions may be subjected to resection, mutagenesis, etc. Thus, transitions, transversions, deletions, and insertions may be performed on the naturally occurring sequence.

[94] Heterologous nucleic acid constructs may include the coding sequence for an protease inhibitor, or a variant, fragment or splice variant thereof: (i) in isolation; (ii) in combination with additional coding sequences; such as fusion protein or signal peptide coding sequences, where the PI coding sequence is the dominant coding sequence; (iii) in combination with non-coding sequences, such as introns and control elements, such as promoter and terminator elements or 5' and/or 3' untranslated regions, effective for expression of the coding sequence in a suitable host; and/or (iv) in a vector or host environment in which the PI coding sequence is a heterologous gene.

[95] A heterologous nucleic acid containing the appropriate nucleic acid coding sequence, as described above, together with appropriate promoter and control sequences, may be employed to transform filamentous fungal cells to permit the cells to express a protease inhibitor or variant thereof.

[96] In one aspect of the present invention, a heterologous nucleic acid construct is employed to transfer a PI-encoding nucleic acid sequence into a cell *in vitro*, with established cell lines preferred. Preferably, cell lines that are to be used as production hosts have the nucleic acid sequences of this invention stably integrated. It follows that any method effective to generate stable transformants may be used in practicing the invention.

[97] In one aspect of the present invention, the first and second expression cassettes may be present on a single vector or on separate vectors.

[98] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, and recombinant DNA, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, "Molecular Cloning: A Laboratory Manual", Second Edition (Sambrook, Fritsch & Maniatis, 1989), "Animal Cell Culture" (R. I. Freshney, ed., 1987); and "Current Protocols in Molecular Biology" (F. M. Ausubel *et al.*, eds., 1987). All patents, patent applications, articles and publications mentioned herein, both *supra* and *infra*, are hereby expressly incorporated herein by reference.

[99] In addition to a promoter sequence, the expression cassette should also contain a transcription termination region downstream of the structural gene to provide for efficient termination. The termination region may be obtained from the same gene as the promoter sequence or may be obtained from different genes, also within the knowledge of one skilled in the art.

[100] The particular expression vector used to transport the genetic information into the cell is not particularly critical. Any of the conventional vectors used for expression in eukaryotic or prokaryotic cells may be used. Standard bacterial expression vectors include bacteriophages λ and M13, as well as plasmids such as pBR322 based plasmids, pSKF, pET23D, and fusion expression systems such as MBP, GST, and LacZ. Epitope tags can also be added to recombinant proteins to provide convenient methods of isolation, e.g., c-myc.

[101] The elements that are typically included in expression vectors also include a replicon, a gene encoding antibiotic resistance to permit selection of bacteria that harbor recombinant plasmids, and unique restriction sites in nonessential regions of the plasmid to allow insertion of heterologous sequences. The particular antibiotic resistance gene chosen is not critical, any of the many resistance genes known in the art are suitable.

C. Host Cells and Culture Conditions.

[102] The present invention provides cell lines comprising cells which have been modified, selected and cultured in a manner effective to result in expression of a protease inhibitor and variants thereof.

[103] Examples of parental cell lines which may be treated and/or modified for PI expression include, but are not limited to, filamentous fungal cells. Examples of appropriate primary cell types for use in practicing the invention include, but are not limited to, *Aspergillus* and *Trichoderma*.

[104] Protease inhibitor expressing cells are cultured under conditions typically employed to culture the parental cell line. Generally, cells are cultured in a standard medium containing physiological salts and nutrients, such as standard RPMI, MEM, IMEM or DMEM, typically supplemented with 5-10% serum, such as fetal bovine serum. Culture conditions are also standard, *e.g.*, cultures are incubated at 37°C in stationary or roller cultures until desired levels of protease inhibitor expression are achieved.

[105] Preferred culture conditions for a given cell line may be found in the scientific literature and/or from the source of the cell line such as the American Type Culture Collection (ATCC; "http://www.atcc.org/"). Typically, after cell growth has been established, the cells are exposed to conditions effective to cause or inhibit the expression of a protease inhibitor and variants thereof.

[106] In the preferred embodiments, where a PI coding sequence is under the control of an inducible promoter, the inducing agent, *e.g.*, a carbohydrate, metal salt or antibiotics, is added to the medium at a concentration effective to induce protease inhibitor expression.

D. Introduction Of A Protease Inhibitor-Encoding Nucleic Acid Sequence Into Host Cells.

[107] The methods of transformation used may result in the stable integration of all or part of the transformation vector into the genome of the filamentous fungus. However, transformation resulting in the maintenance of a self-replicating extra-chromosomal transformation vector is also contemplated.

[108] The invention further provides cells and cell compositions which have been genetically modified to comprise an exogenously provided PI-encoding nucleic acid sequence. A parental cell or cell line may be genetically modified (*i.e.*, transduced, transformed or transfected) with a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc, as further described above. In a preferred embodiment, a plasmid is used to transfect a filamentous fungal cell. The transformations may be sequential or by co-transformation.

[109] Various methods may be employed for delivering an expression vector into cells *in vitro*. Methods of introducing nucleic acids into cells for expression of heterologous nucleic acid sequences are also known to the ordinarily skilled artisan, including, but not limited to electroporation; nuclear microinjection or direct microinjection into single cells; protoplast fusion with intact cells; use of polycations, *e.g.*, polybrene or polyornithine; or PEG. membrane fusion with liposomes, lipofectamine or lipofection-mediated transfection; high velocity bombardment with DNA-coated microprojectiles; incubation with calcium phosphate-

DNA precipitate; DEAE-Dextran mediated transfection; infection with modified viral nucleic acids; *Agrobacterium*-mediated transfer of DNA; and the like. In addition, heterologous nucleic acid constructs comprising a PI-encoding nucleic acid sequence can be transcribed *in vitro*, and the resulting RNA introduced into the host cell by well-known methods, *e.g.*, by injection.

[110] Following introduction of a heterologous nucleic acid construct comprising the coding sequence for a protease inhibitor, the genetically modified cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying expression of a PI-encoding nucleic acid sequence. The culture conditions, such as temperature, pH and the like, are those previously used for the host cell selected for expression, and will be apparent to those skilled in the art.

[111] The progeny of cells into which such heterologous nucleic acid constructs have been introduced are generally considered to comprise the PI-encoding nucleic acid sequence found in the heterologous nucleic acid construct.

E. Fungal Expression

[112] Appropriate host cells include filamentous fungal cells. The "filamentous fungi" of the present invention, which serve both as the expression hosts and the source of the first and second nucleic acids, are eukaryotic microorganisms and include all filamentous forms of the subdivision Eumycotina, Alexopoulos, C.J. (1962), Introductory Mycology, New York: Wiley. These fungi are characterized by a vegetative mycelium with a cell wall composed of chitin, glucans, and other complex polysaccharides. The filamentous fungi of the present invention are morphologically, physiologically, and genetically distinct from yeasts. Vegetative growth by filamentous fungi is by hyphal elongation. In contrast, vegetative growth by yeasts such as *S. cerevisiae* is by budding of a unicellular thallus. Illustrations of differences between *S. cerevisiae* and filamentous fungi include the inability of *S. cerevisiae* to process *Aspergillus* and *Trichoderma* introns and the inability to recognize many transcriptional regulators of filamentous fungi (Innis, M.A. *et al.* (1985) Science, 228, 21-26).

[113] Various species of filamentous fungi may be used as expression hosts including the following genera: *Aspergillus, Trichoderma, Neurospora, Penicillium, Cephalosporium, Achlya, Phanerochaete, Podospora, Endothia, Mucor, Fusarium, Humicola,* and *Chrysosporium*.. Specific expression hosts include *A. nidulans*, (Yelton, M., *et al.* (1984) Proc. Natl. Acad. Sci. USA, 81, 1470-1474; Mullaney, E.J. *et al.* (1985) Mol. Gen. Genet. 199, 37-45; John, M.A. and J.F. Peberdy (1984) Enzyme Microb. Technol. 6, 386-389; Tilburn, *et al.* (1982) Gene 26, 205-221; Ballance, D.J. et al., (1983) Biochem. Biophys. Res.

Comm. 112, 284-289; Johnston, I.L. *et al.* (1985) EMBO J. 4, 1307-1311) *A. niger*, (Kelly, J.M. and M. Hynes (1985) EMBO 4, 475-479) *A. niger* var. *awamori*, e.g., NRRL 3112, ATCC 22342, ATCC 44733, ATCC 14331 and strain UVK 143f, *A. oryzae*, e.g., ATCC 11490, *N. crassa* (Case, M.E. *et al.* (1979) Proc. Natl. Acad. Sci. USA 76, 5259-5263; Lambowitz U.S. Patent No. 4,486,553; Kinsey, J.A. and J.A. Rambosek (1984) *Molecular and Cellular Biology 4*, 117-122; Bull, J.H. and J.C. Wooton (1984) Nature 310, 701-704), *Trichoderma reesei*, e.g. NRRL 15709, ATCC 13631, 56764, 56765, 56466, 56767, and *Trichoderma viride*, e.g., ATCC 32098 and 32086. A preferred expression host is *A. niger* var. *awamori* in which the gene encoding the major secreted aspartyl protease has been deleted. The production of this preferred expression host is described in United States Patent Application Serial No. 214,237 filed July 1, 1988, expressly incorporated herein by reference.

[114] During the secretion process in fungi, which are eukaryotes, the secreted protein crosses the membrane from the cytoplasm into the lumen of the endoplasmic reticulum (ER). It is here that the protein folds and disulphide bonds are formed. Chaperone proteins such as BiP and proteins like protein disulphide isomerase assist in this process. It is also at this stage where sugar chains are attached to the protein to produce a glycosylated protein. Sugars are typically added to asparagine residues as N-linked glycosylation or to serine or threonine residues as O-linked glycosylation. Correctly folded and glycosylated proteins pass from the ER to the Golgi apparatus where the sugar chains are modified and where the KEX2 or KEXB protease of yeast and fungi resides. The N-linked glycosylation added to secreted proteins produced in fungi differs from that added by mammalian cells.

[115] Protease inhibitor and variants thereof produced by the filamentous fungal host cells may be either glycosylated or non-glycosylated (i.e., aglycosylated or deglycosylated). Because the fungal glycosylation pattern differs from that produced by mammalian cells, the protease inhibitor may be treated with an enzyme to deglycosylate the protease inhibitor Enzymes useful for such N-linked deglycosylation are endoglycosidase H, endoglycosidase F1, endoglycosidase F2, endoglycosidase A, PNGase F, PNGase A, and PNGase At. Enzymes useful for such O-linked deglycosylation are exoglycosidases, specifically alpha-mannosidases (e.g. alpha-Mannosidase (*Aspergillus saito,i*GKX-5009), alpha(1-2, 3, 6)-Mannosidase (Jack bean, GKX-5010) alpha-Mannosidase/MANase VI (recombinant from *Xanthomonas manihoti,* GKX80070) all from Glyko (Prozyme), San Leandro, California).

[116] We have surprisingly found that high levels of a protease inhibitor and variants thereof can be made in fungi when fused to a native secreted protein. From the information

provided above it is clear that the protease inhibitor and variants thereof would be expected to assemble in the ER when glucoamylase was still attached to the N-termini. This would produce a large protein of greater than 56 kD. The glucoamylase would not be expected to be cleaved from the desired protein when it passed through the Golgi apparatus without further modification.

[117] Using the present inventive methods and host cells, we have attained surprising levels of expression. The system utilized herein has achieved levels of expression and secretion of greater than 0.5 g/l of protease inhibitor. .

[118] After the expression vector is introduced into the cells, the transfected cells are cultured under conditions favoring expression of gene encoding the desired protein. Large batches of transformed cells can be cultured as described above. Finally, product is recovered from the culture using techniques known in the art.

CHAPERONES

[119] As noted above, the folding and glycosylation of the secretory proteins in the ER is assisted by numerous ER-resident proteins called chaperones. The chaperones like Bip (GRP78), GRP94 or yeast Lhs1p help the secretory protein to fold by binding to exposed hydrophobic regions in the unfolded states and preventing unfavourable interactions (Blond-Elguindi et al., 1993, Cell 75:717-728). The chaperones are also important for the translocation of the proteins through the ER membrane. The foldase proteins like protein disulphide isomerase (pdi) and its homologs and prolyl-peptidyl cis-trans isomerase assist in formation of disulphide bridges and formation of the right conformation of the peptide chain adjacent to proline residues, respectively.

[120] In one aspect of the invention the host cells are transformed with an expression vector encoding a chaperone. The chaperone is selected from the group consisting of pdiA and prpA.

FERMENTATION PARAMETERS

[121] The invention relies on fermentation procedures for culturing fungi. Fermentation procedures for production of heterologous proteins are known per se in the art. For example, proteins can be produced either by solid or submerged culture, including batch, fed-batch and continuous-flow processes.

[122] Culturing is accomplished in a growth medium comprising an aqueous mineral salts medium, organic growth factors, the carbon and energy source material, molecular oxygen, and, of course, a starting inoculum of one or more particular microorganism species to be employed.

[123] In addition to the carbon and energy source, oxygen, assimilable nitrogen, and an inoculum of the microorganism, it is necessary to supply suitable amounts in proper proportions of mineral nutrients to assure proper microorganism growth, maximize the assimilation of the carbon and energy source by the cells in the microbial conversion process, and achieve maximum cellular yields with maximum cell density in the fermentation media.

[124] The composition of the aqueous mineral medium can vary over a wide range, depending in part on the microorganism and substrate employed, as is known in the art. The mineral media should include, in addition to nitrogen, suitable amounts of phosphorus, magnesium, calcium, potassium, sulfur, and sodium, in suitable soluble assimilable ionic and combined forms, and also present preferably should be certain trace elements such as copper, manganese, molybdenum, zinc, iron, boron, and iodine, and others, again in suitable soluble assimilable form, all as known in the art.

[125] The fermentation reaction is an aerobic process in which the molecular oxygen needed is supplied by a molecular oxygen-containing gas such as air, oxygen-enriched air, or even substantially pure molecular oxygen, provided to maintain the contents of the fermentation vessel with a suitable oxygen partial pressure effective in assisting the microorganism species to grow in a thriving fashion. In effect, by using an oxygenated hydrocarbon substrate, the oxygen requirement for growth of the microorganism is reduced. Nevertheless, molecular oxygen must be supplied for growth, since the assimilation of the substrate and corresponding growth of the microorganisms, is, in part, a combustion process.

[126] Although the aeration rate can vary over a considerable range, aeration generally is conducted at a rate which is in the range of about 0.5 to 10, preferably about 0.5 to 7, volumes (at the pressure employed and at 25°C.) of oxygen-containing gas per liquid volume in the fermentor per minute. This amount is based on air of normal oxygen content being supplied to the reactor, and in terms of pure oxygen the respective ranges would be about 0.1 to 1.7, or preferably about 0.1 to 1.3, volumes (at the pressure employed and at 25°C.) of oxygen per liquid volume in the fermentor per minute.

[127] The pressure employed for the microbial conversion process can range widely. Pressures generally are within the range of about 0 to 50 psig, presently preferably about 0 to 30 psig, more preferably at least slightly over atmospheric pressure, as a balance of equipment and operating cost versus oxygen solubility achieved. Greater than atmospheric pressures are advantageous in that such pressures do tend to increase a dissolved oxygen

concentration in the aqueous ferment, which in turn can help increase cellular growth rates. At the same time this is balanced by the fact that high atmospheric pressures do increase equipment and operating costs.

[128] The fermentation temperature can vary somewhat, but for filamentous fungi such as *Aspergillus niger var. awamori* the temperature generally will be within the range of about 20°C to 40°C, generally preferably in the range of about 28°C to 37°C, depending on the strain of microorganism chosen.

[129] The microorganisms also require a source of assimilable nitrogen. The source of assimilable nitrogen can be any nitrogen-containing compound or compounds capable of releasing nitrogen in a form suitable for metabolic utilization by the microorganism. While a variety of organic nitrogen source compounds, such as protein hydrolysates, can be employed, usually cheap nitrogen-containing compounds such as ammonia, ammonium hydroxide, urea, and various ammonium salts such as ammonium phosphate, ammonium sulfate, ammonium pyrophosphate, ammonium chloride, or various other ammonium compounds can be utilized. Ammonia gas itself is convenient for large scale operations, and can be employed by bubbling through the aqueous ferment (fermentation medium) in suitable amounts. At the same time, such ammonia can also be employed to assist in pH control.

[130] The pH range in the aqueous microbial ferment (fermentation admixture) should be in the exemplary range of about 2.0 to 8.0. With filamentous fungi, the pH normally is within the range of about 2.5 to 8.0; with *Aspergillus niger var. awamori*, the pH normally is within the range of about 4.5 to 5.5. pH range preferences for certain microorganisms are dependent on the media employed to some extent, as well as the particular microorganism, and thus change somewhat with change in media as can be readily determined by those skilled in the art.

[131] While the average retention time of the fermentation admixture in the fermentor can vary considerably, depending in part on the fermentation temperature and culture employed, generally it will be within the range of about 24 to 500 hours, preferably presently about 24 to 400 hours.

[132] Preferably, the fermentation is conducted in such a manner that the carbon-containing substrate can be controlled as a limiting factor, thereby providing good conversion of the carbon-containing substrate to cells and avoiding contamination of the cells with a substantial amount of unconverted substrate. The latter is not a problem with water-soluble substrates, since any remaining traces are readily washed off. It may be a problem,

however, in the case of non-water-soluble substrates, and require added product-treatment steps such as suitable washing steps.

[133] As described above, the time to reach this limiting substrate level is not critical and may vary with the particular microorganism and fermentation process being conducted. However, it is well known in the art how to determine the carbon source concentration in the fermentation medium and whether or not the desired level of carbon source has been achieved.

[134] Although the fermentation can be conducted as a batch or continuous operation, fed batch operation is generally preferred for ease of control, production of uniform quantities of products, and most economical uses of all equipment.

[135] If desired, part or all of the carbon and energy source material and/or part of the assimilable nitrogen source such as ammonia can be added to the aqueous mineral medium prior to feeding the aqueous mineral medium to the fermentor.

[136] Each of the streams introduced into the reactor preferably is controlled at a predetermined rate, or in response to a need determinable by monitoring such as concentration of the carbon and energy substrate, pH, dissolved oxygen, oxygen or carbon dioxide in the off-gases from the fermentor, cell density measurable by light transmittancy, or the like. The feed rates of the various materials can be varied so as to obtain as rapid a cell growth rate as possible, consistent with efficient utilization of the carbon and energy source, to obtain as high a yield of microorganism cells relative to substrate charge as possible, but more importantly to obtain the highest production of the desired protein per unit volume.

[137] In either a batch, or the preferred fed batch operation, all equipment, reactor, or fermentation means, vessel or container, piping, attendant circulating or cooling devices, and the like, are initially sterilized, usually by employing steam such as at about 121°C for at least about 15 minutes. The sterilized reactor then is inoculated with a culture of the selected microorganism in the presence of all the required nutrients, including oxygen, and the carbon-containing substrate. The type of fermentor employed is not critical, though presently preferred is operation under 15L Biolafitte (Saint-Germain-en-Laye, France).

PROTEIN SEPARATIONS

[138] Once the desired protein is expressed and, optionally, secreted recovery of the desired protein may be necessary. The present invention provides methods of separating a desired protein from its fusion analog. It is specifically contemplated that the methods described herein are useful for the separation of proteinase inhibitor and variants from the fusion analog.

[139] The collection and purification of the desired protein from the fermentation broth can also be done by procedures known per se in the art. The fermentation broth will generally contain cellular debris, including cells, various suspended solids and other biomass contaminants, as well as the desired protein product, which are preferably removed from the fermentation broth by means known in the art.

[140] Suitable processes for such removal include conventional solid-liquid separation techniques such as, e.g., centrifugation, filtration, dialysis, microfiltration, rotary vacuum filtration, or other known processes, to produce a cell-free filtrate. It may be preferable to further concentrate the fermentation broth or the cell-free filtrate prior to crystallization using techniques such as ultrafiltration, evaporation or precipitation.

[141] Precipitating the proteinaceous components of the supernatant or filtrate may be accomplished by means of a salt, e.g., ammonium sulfate or adjust pH to 2 to 3 and then heat treatment of the broth at 80°C for 2 hours, followed by purification by a variety of chromatographic procedures, e.g., ion exchange chromatography, affinity chromatography or similar art recognized procedures.

[142] When the expressed desired polypeptide is secreted the polypeptide may be purified from the growth media. Preferably the expression host cells are removed from the media before purification of the polypeptide (e.g. by centrifugation).

[143] When the expressed recombinant desired polypeptide is not secreted from the host cell, the host cell is preferably disrupted and the polypeptide released into an aqueous "extract" which is the first stage of purification. Preferably the expression host cells are collected from the media before the cell disruption (e.g. by centrifugation).

[144] The cell disruption may be performed by conventional techniques such as by lysozyme or beta-glucanase digestion or by forcing the cells through high pressure. See (Robert K. Scobes, Protein Purification, Second edition, Springer-Verlag) for further description of such cell disruption techniques.

[145] The addition of six histidine residues, i.e., a His Tag, to the C-terminus may also aid in the purification of the desired protein and its fusion analog. Use of the His tag as a purification aid is well known in the art. See, for example, Hengen (1995) TIBS 20(7):285-286. The 6x his-tagged proteins are easily purified using Immobilized Metal ion Affinity Chromatography (IMAC).

[146] It is specifically contemplated that protease inhibitors and variants thereof may be purified from an aqueous protein solution, e.g., whole cell fermentation broth or clarified

broth, using a combination of hydrophobic charge induction chromatography (HCIC). HCIC provided an ability to separate the desired protein from the broth and from its fusion analog.

UTILITY

[147] For some applications of desired proteins it is of high importance that the protease inhibitors are extremely pure, e.g. having a purity of more than 99%. This is particularly true whenever the desired protein is to be used as a therapeutic, but is also necessary for other applications. The methods described herein provide a way of producing substantially pure desired proteins. The desired proteins described herein are useful in pharmaceutical and personal care compositions.

[148] In the experimental disclosure which follows, the following abbreviations apply: eq (equivalents); M (Molar); μM (micromolar); N (Normal); mol (moles); mmol (millimoles); μmol (micromoles); nmol (nanomoles); g (grams); mg (milligrams); kg (kilograms); μg (micrograms); L (liters); ml (milliliters); μl (microliters); cm (centimeters); mm (millimeters); μm (micrometers); nm (nanometers); ° C. (degrees Centigrade); h (hours); min (minutes); sec (seconds); msec (milliseconds); Ci (Curies) mCi (milliCuries); μCi (microCuries); TLC (thin layer achromatography); Ts (tosyl); Bn (benzyl); Ph (phenyl); Ms (mesyl); Et (ethyl), Me (methyl). PI (proteinase inhibitor), BBI (Bowman-Birk inhibitor), STI (Soybean Trypsin inhibitor).

EXAMPLES

[149] The present invention is described in further detain in the following examples which are not in any way intended to limit the scope of the invention as claimed. The attached Figures are meant to be considered as integral parts of the specification and description of the invention. All references cited are herein specifically incorporated by reference for all that is described therein. The following examples are offered to illustrate, but not to limit the claimed invention.

Example 1
Cloning of DNA encoding the Soybean Trypsin Inhibitor

[150] This example illustrates the development of an expression vector for STI.

[151] In general, the gene encoding the desired protein was fused to the DNA encoding the linker region of glucoamylase with an engineered kexB cleavage site (NVISKR) via an *Nhe*I restriction enzyme site at the N-terminal and a *BstE*II restriction enzyme site at the C-terminal following the STI stop codon, TAG. The gene encoding the soybean STI was

synthesized by MCLAB (South San Francisco, California) *in vitro* as a DNA fragment containing two restriction sites, a kexB cleavage site and three glycine residues at N-terminal end and six histidine residues at C-terminal end. (SEQ ID NO:3, gene shown in Figure 2). All PCR-generated DNA fragments used herein were initially cloned into the pCRII-TOPO vector (Invitrogen, Carlsbad, CA). *E. coli* [One Shot® TOP10 cells from Invitrogen], was used for routine plasmid isolation and plasmid maintenance. The *Nhe*I and *BstE*II sites were used to excise the PCR product from the pCRII-TOPO vector, and the resulting DNA fragment was then ligated into the expression vector, pSL1180-GAMpR-2 (see Figure 5) The expression vector, pSL1180-GAMpR2, contains the *Aspergillus niger* glucoamylase promoter, the glucoamylase catalytic domain and the terminator region. The expression plasmid also contains the *A. niger* pyrG gene as the selection marker. Thus, detection of transformants with the expression cassette is by growth on uridine-deficient medium.

[152] The gene encoding the STI peptide (for amino acid sequence: Figure 4A, SEQ ID NO:10; for nucleotide sequence: Figure 2 and SEQ ID NO:6) was synthesized and cloned into pCRII-TOPO vector (Invitrogen) by MCLAB. The *Nhe*I to *BstE*II fragment was release from the plasmid by restriction digestion and the DNA fragment was extracted from an agarose gel and cloned into pSLGAMpR2, a glucoamylase- chymosin expression vector which is described in detail in WO 9831821. to create expression plasmid pSLGAMpR2-SBTI/nonopti (Q110).

[153] The expression plasmid was transformed into dgr246ΔGAP:pyr2-. This strain is derived from strain dgr246 P2 which has the *pepA* gene deleted, is *pyrG* minus and has undergone several rounds of mutagenesis and screening or selection for improved production of a heterologous gene product (Ward, M. et al., 1993, Appl. Microbiol. Biotech. 39:738-743 and references therein). To create strain dgr246ΔGAP:pyr2- the *glaA* (glucoamylase) gene was deleted in strain dgr246 P2 using exactly the same deletion plasmid (pΔGAM NB-Pyr) and procedure as reported by Fowler, T. et al (1990) Curr. Genet. 18:537-545. Briefly, the deletion was achieved by transformation with a linear DNA fragment having *glaA* flanking sequences at either end and with part of the promoter and coding region of the *glaA* gene replaced by the *Aspergillus nidulans pyrG* gene as selectable marker. Transformants in which the linear fragment containing the *glaA* flanking sequences and the *pyrG* gene had integrated at the chromosomal *glaA* locus were identified by Southern blot analysis. This change had occurred in transformed strain dgr246ΔGAP. Spores from this transformant were plated onto medium containing fluoroorotic acid and spontaneous resistant mutants were obtained as described by van Hartingsveldt, W. et al.

(1987) Mol. Gen. Genet. 206:71-75. One of these, dgr246ΔGAP:pyr2-, was shown to be a uridine auxotroph strain which could be complemented by transformation with plasmids bearing a wild-type *pyrG* gene.

[154] The *Aspergillus* transformation protocol was a modification of the Campbell method (Campbell et at. (1989). Curr. Genet. 16:53-56). All solutions and media were either autoclaved or filter sterilized through a 0.2 micron filter. Spores of *A. niger* var. *awamori* were harvested from complex media agar (CMA) plates. CMA contained 20 g/l dextrose, 20 g/l Difco Brand malt extract, 1 g/l Bacto Peptone, 20 g/l Bacto agar, 20 ml/l of 100 mg/ml arginine and 20 ml/l of 100 mg/ml uridine. An agar plug of approximately 1.5 cm square of spores was used to inoculate 100 mls of liquid CMA (recipe as for CMA except that the Bacto agar was omitted). The flask was incubated at 37°C on a shaker at 250-275 rpm, overnight. The mycelia were harvested through sterile Miracloth (Calbiochem, San Diego, CA, USA) and washed with 50 mls of Solution A (0.8M $MgSO_4$ in 10 mM sodium phosphate, pH 5.8). The washed mycelia were placed in a sterile solution of 300 mg of beta-D-glucanase (Interspex Products, San Mateo, CA) in 20 mls of solution A. This was incubated at 28° to 30°C at 200 rpm for 2 hour in a sterile 250 ml plastic bottle (Corning Inc, Corning, New York). After incubation, this protoplasting solution was filtered through sterile Miracloth into a sterile 50 ml conical tube (Sarstedt, USA). The resulting liquid containing protoplasts was divided equally amongst two 50 ml conical tubes. Forty ml of solution B (1.2 M sorbitol, 50 mM $CaCl_2$, 10 mM Tris, pH7.5) were added to each tube and centrifuged in a table top clinical centrifuge (Damon IEC HN SII centrifuge) at full speed for 5 minutes. The supernatant from each tube was discarded and 20 mls of fresh solution B was added to one tube, mixed, then poured into the next tube until all the pellets were resuspended. The tube was then centrifuged for 5 minutes. The supernatant was discarded, 20 mls of fresh solution B was added, the tube was centrifuged for 5 minutes. The wash occurred one last time before resuspending the washed protoplasts in solution B at a density of 0.5-1.0 X 10^7 protoplasts/l00ul. To each 100 ul of protoplasts in a sterile 15 ml conical tube (Sarstedt, USA), 10 ul of the transforming plasmid DNA was added. To this, 12.5 ul of solution C (50% PEG 4000, 50 mM $CaCl_2$, 10 mM Tris, pH 7.5) was added and the tube was placed on ice for 20 minutes. One ml of solution C was added and the tube was removed from the ice to room temperature and shaken gently. Two ml of solution B was added immediately to dilute solution C. The transforming mix was added equally to 3 tubes of melted MMS overlay (6 g/l $NaNO_3$, 0.52 g/l KCl, 1.52 g/l KH_2PO_4, 218.5 g/l D-sorbitol, 1.0 ml/l trace elements-LW, 10 g/l SeaPlaque agarose (FMC Bioproducts, Rook1and, Maine, USA) 20 ml/l 50% glucose, 2.5

ml/l 20% $MgSO_4.7H_20$, pH to 6.5 with NaOH) that were stored in a 45°C water bath. Trace elements-LW consisted of 1 g/l $FeSO_4.7H_2O$, 8.8 g/l $ZnSO_4.7H_2O$, 0.4 g/l $CuSO_4.5H_2O$, 0.15 g/l $MnSO_4.4H_2O$, 0.1 g $Na_2B_4O_7.10H_2O$, 50 mg/l $(NH_4)_6Mo_7O_{24}.4H_2O$, 250 mls H2O, 200 ul/l concentrated HCl. The melted overlays with the transformation mix were immediately poured onto 3 MMS plates (same as MMS overlay recipe with the exception of 20 g/l of Bacto agar instead of 10 g/l of SeaPlaque agarose) that had been supplemented with 333 ul/plate of 100 mg/ml of arginine added directly on top of the agar plate. After the agarose solidified, the plates were incubated at 30°C until transformants grew.

[155] The sporulating transformants were picked off with a sterile toothpick onto a plate of minimal media + glucose (MM). MM consisted of 6 g/l $NaNO_3$, 0.52 g/l KC1, 1.52 g/l KH_2PO_4, 1 ml/l Trace elements-LW, 20 g/l Bacto agar, pH to 6.5 with NaOH, 25 ml/l of 40 % glucose, 2.5 ml/l of 20% $MgSO_4.7H_2O$ and 20 ml/l of 100 mg/ml arginine. Once the transformants grew on MM they were transferred to CMA plates.

[156] A 1.5 cm square agar plug from a plate culture of each transformant was added to 50 mls, in a 250 ml shake flask, of production medium called Promosoy special. This medium had the following components: 70 g/l sodium citrate, 15 g/l $(NH_4)_2$ $S0_4$, 1 g/l $NaH_2PO_4.H_2O$, 1 g/l $MgSO_4$, 1 ml Tween 80, pH to 6.2 with NaOH, 2 ml/l Mazu DF60-P, 45 g/l Promosoy 100 (Central Soya, Fort Wayne, IN), 120 g/l maltose. The production media flasks were incubated at 30°C, 200 rpm for 5 days and supernatant samples were harvested. Transformants were assayed for protein production on SDS gel to select the transformants based on the amount of protein produced. Broth from the top transformants were assayed for Trypsin or chymotrypsin inhibition activity

[157] A 1.5 cm square agar plug from a plate culture of each transformant was also added to 50 mls, in a 250 ml shake flask, of production medium called modified CSS. This medium had the following components: 50g/l Corn Streep Solids, 1g/l NaH2PO4*H2O, 0.5g/l MgSO4 (anhydrous), 50g/l Staley 7350 (55%) and 8g/l Na Citrate. The production media flasks were incubated at 36°C, 200 rpm for 3 days and supernatant samples were harvested and assayed for protein production on SDS gel. Broth from the top transformants were assayed for Trypsin or Chymotrypsin inhibition activity.

Example 2
Codon optimization of the DNA encoding the Soybean Trypsin Inhibitor

[158] The following example details how the STI-encoding DNA was altered for optimized expression in a filamentous fungi.

[159] The codons from the synthetic gene (the starting material in Example 1 that was synthesized by MCLAB) were then optimized according to the codon usage of highly expressed proteins in *Aspergillus*. Basically, proteins that expressed well such as glucoamylase, alpha-amylase and prochymosin were compared to proteins that did not express well in *Aspergillus* such as human NEP and DPP4. See Table I. The codon usage table for both types of protein expressions in *Aspergillus* is in Table II.

Table II

Codon	AA	NEP coding	DPP4		SCCE	bovine prochymosin	chymotrypsin	Her2 Light chain	Stachybotrys oxidase B	stachybotrys oxidase A	glucoamylase w/out starch binding domain
gca *	Ala(A)	19	15		1	0	1	1	2	4	10
gcc	Ala(A)	12	6		8	15	17	8	19	23	18
gcg	Ala(A)	1	2		0	1	1	2	1	0	9
gcu	Ala(A)	18	12		2	1	3	3	25	21	20
---	Ala(A)	50	35		11	17	22	14	47	48	57
aga *	Arg(R)	15	16		3	1	0	2	2	2	1
agg	Arg(R)	5	8		4	6	4	1	4	5	1
cga	Arg(R)	6	1		0	1	0	0	7	3	3
cgc	Arg(R)	2	1		4	1	1	2	12	11	6
cgg	Arg(R)	1	3		0	0	0	0	0	0	2
cgu	Arg(R)	4	1		0	0	1	4	7	8	4
---	Arg(R)	33	30		11	9	6	9	32	29	17
aac	Asn(N)	20	16		1	11	6	5	26	27	17
aau *	Asn(N)	36	23		9	4	3	1	5	6	6
---	Asn(N)	56	39		10	15	9	6	31	33	23
gac	Asp(D)	13	16		10	20	14	5	24	20	17
gau *	Asp(D)	28	27		4	2	1	5	18	19	18
---	Asp(D)	41	43		14	22	15	10	42	39	35
ugc	Cys(C)	7	4		10	3	8	4	1	0	6
ugu	Cys(C)	5	8		2	3	2	1	0	1	2
---	Cys(C)	12	12		12	6	10	5	1	1	8
caa *	Gln(Q)	14	15		3	1	1	3	2	6	4
cag	Gln(Q)	17	15		7	24	8	12	14	13	11
---	Gln(Q)	31	30		10	25	9	15	16	19	15
gaa *	Glu(E)	36	31		4	2	1	1	2	4	7
gag	Glu(E)	17	9		2	12	5	8	38	42	11
---	Glu(E)	53	40		6	14	6	9	40	46	18

Codon	AA	NEP c ding	DPP4		SCCE	bovine prochymosin	chymotrypsin	Her2 Light chain	Stachybotrys oxidas B	stachybotrys oxidase A	glucoamylase w/out starch binding domain
gga *	Gly(G)	15	20		5	1	1	2	10	10	5
ggc	Gly(G)	12	7		11	15	14	5	17	20	20
ggg	Gly(G)	7	6		1	12	7	0	0	1	4
ggu	Gly(G)	7	7		4	3	1	4	17	11	12
---	Gly(G)	41	40		21	31	23	11	44	42	41
cac	His(H)	5	8		5	4	3	2	11	13	4
cau	His(H)	4	11		2	2	0	1	2	4	0
---	His(H)	9	19		7	6	3	3	13	17	4
aua	Ile(I)	9	13		1	1	1	0	0	0	0
auc	Ile(I)	10	12		3	19	9	5	19	16	8
auu *	Ile(I)	26	22		2	2	2	1	6	12	11
---	Ile(I)	45	47		6	22	12	6	25	28	19
cua *	Leu(L)	5	6		0	0	0	0	0	0	2
cuc	Leu(L)	7	8		8	5	4	3	13	18	16
cug	Leu(L)	9	15		10	23	15	10	14	16	15
cuu	Leu(L)	15	7		0	1	0	0	11	12	3
uua *	Leu(L)	7	10		1	0	0	0	0	0	0
uug *	Leu(L)	16	9		1	0	0	1	5	3	6
---	Leu(L)	59	55		20	29	19	14	43	49	42
aaa *	Lys(K)	32	27		3	6	1	5	0	0	0
aag	Lys(K)	17	10		13	9	14	9	7	19	11
---	Lys(K)	49	37		16	15	15	14	7	19	11
aug	Met(M)	14	14		6	8	2	1	17	12	3
---	Met(M)	14	14		6	8	2	1	17	12	3
uuc	Phe(F)	12	14		3	13	6	9	24	21	17
uuu *	Phe(F)	16	17		1	6	1	0	3	6	2
---	Phe(F)	28	31		4	19	7	9	27	27	19
cca *	Pro(P)	9	14		4	1	0	2	1	6	0
ccc	Pro(P)	6	2		7	11	8	6	20	17	8
ccg	Pro(P)	0	1		1	3	0	2	4	2	6
ccu	Pro(P)	7	10		2	1	5	2	21	16	3
---	Pro(P)	22	27		14	16	13	12	46	41	17
agc	Ser(S)	7	12		2	13	6	14	8	7	19
agu *	Ser(S)	7	14		2	4	1	1	2	1	10
uca *	Ser(S)	11	17		3	1	0	0	2	2	2
ucc	Ser(S)	7	10		9	9	14	11	11	8	16
ucg	Ser(S)	0	1		1	4	0	2	4	3	11
ucu	Ser(S)	11	10		1	4	2	5	9	8	15

Codon	AA	NEP coding	DPP4		SCCE	bovine prochymosin	chymotrypsin	Her2 Light chain	Stachybotrys oxidas B	stachybotrys oxidase A	glucoamylas w/out starch binding domain
---	Ser(S)	43	64		18	35	23	33	36	29	73
uaa	Ter(.)	0	0		1	0	0	0	0	1	0
uag	Ter(.)	0	1		0	0	0	0	0	0	0
uga	Ter(.)	1	0		0	0	1	1	0	0	0
---	Ter(.)	1	1		1	0	1	1	0	1	0
aca *	Thr(T)	10	21		1	5	4	2	2	1	4
acc	Thr(T)	9	6		9	13	13	14	12	16	28
acg	Thr(T)	1	1		3	2	0	2	1	1	8
acu	Thr(T)	10	17		5	4	0	3	16	12	14
---	Thr(T)	30	45		18	24	17	21	31	30	54
ugg	Trp(W)	14	20		5	4	8	2	11	14	15
---	Trp(W)	14	20		5	4	8	2	11	14	15
uac	Tyr(Y)	11	26		4	17	2	9	21	24	16
uau *	Tyr(Y)	22	30		0	5	0	2	4	4	6
---	Tyr(Y)	33	56		4	22	2	11	25	28	22
gua *	Val(V)	5	7		0	2	0	1	0	2	2
guc	Val(V)	9	12		6	7	10	10	17	25	13
gug	Val(V)	13	14		10	14	14	4	8	6	14
guu	Val(V)	10	11		2	3	0	1	21	12	5
---	Val(V)	37	44		18	26	24	16	46	45	34
nnn	???(X)	0	0		0	0	0	0	3	0	0
TOT	AL	701	729		232	365	246	222	583	597	527

[160] It is evident that many codons were not used or not used as often in the genes that expressed well. These codons were found much more frequently in those genes that were not expressed well (indicated with an asterisk in Table II). In the STI gene, we identified several such codons that were not used or not used often by other well expressed proteins and the codons were changed to the codons that are used more often in well expressed proteins. See Tables III and IV.

TABLE III

Codon usage for wild type STI: (without three glycine residues and six histidine residues and the stop codon)

gca	Ala(A)	4	# cag	Gln(Q)	2	# uug	Leu(L)	3	# uaa	Ter(.)	0
gcc	Ala(A)	2	# ---	Gln(Q)	5	# ---	Leu(L)	15	# uag	Ter(.)	0
gcg	Ala(A)	0	# gaa	Glu(E)	7	# aaa	Lys(K)	7	# uga	Ter(.)	0
gcu	Ala(A)	2	# gag	Glu(E)	6	# aag	Lys(K)	3	# ---	Ter(.)	0
---	Ala(A)	8	# ---	Glu(E)	13	# ---	Lys(K)	10	# aca	Thr(T)	3
aga	Arg(R)	4	# gga	Gly(G)	6	# aug	Met(M)	2	# acc	Thr(T)	2
agg	Arg(R)	1	# ggc	Gly(G)	2	# ---	Met(M)	2	# acg	Thr(T)	1
cga	Arg(R)	1	# ggg	Gly(G)	3	# uuc	Phe(F)	4	# acu	Thr(T)	1
cgc	Arg(R)	1	# ggu	Gly(G)	5	# uuu	Phe(F)	5	# ---	Thr(T)	7
cgg	Arg(R)	0	# ---	Gly(G)	16	# ---	Phe(F)	9	# ugg	Trp(W)	2
cgu	Arg(R)	2	# cac	His(H)	0	# cca	Pro(P)	4	# ---	Trp(W)	2
---	Arg(R)	9	# cau	His(H)	2	# ccc	Pro(P)	0	# uac	Tyr(Y)	0
aac	Asn(N)	4	# ---	His(H)	2	# ccg	Pro(P)	1	# uau	Tyr(Y)	4
aau	Asn(N)	5	# aua	Ile(I)	3	# ccu	Pro(P)	5	# ---	Tyr(Y)	4
---	Asn(N)	9	# auc	Ile(I)	5	# ---	Pro(P)	10	# gua	Val(V)	0
gac	Asp(D)	3	# auu	Ile(I)	6	# agc	Ser(S)	1	# guc	Val(V)	0
gau	Asp(D)	14	# ---	Ile(I)	14	# agu	Ser(S)	1	# gug	Val(V)	8
---	Asp(D)	17	# cua	Leu(L)	1	# uca	Ser(S)	3	# guu	Val(V)	6
ugc	Cys(C)	1	# cuc	Leu(L)	3	# ucc	Ser(S)	1	# ---	Val(V)	14
ugu	Cys(C)	3	# cug	Leu(L)	2	# ucg	Ser(S)	1	# nnn	???(X)	0
---	Cys(C)	4	# cuu	Leu(L)	5	# ucu	Ser(S)	4	# TOTAL		181
caa	Gln(Q)	3	# uua	Leu(L)	1	# ---	Ser(S)	11	#		

Table IV

Codon usage for *A. niger* codon optimized STI I: (without three glycine residues and six histidine residues and the stop codon):

gca	Ala(A)	4	# cag	Gln(Q)	2	# uug	Leu(L)	0	# uaa	Ter(.)	0
gcc	Ala(A)	2	# ---	Gln(Q)	5	# ---	Leu(L)	15	# uag	Ter(.)	0
gcg	Ala(A)	0	# gaa	Glu(E)	7	# aaa	Lys(K)	7	# uga	Ter(.)	0
gcu	Ala(A)	2	# gag	Glu(E)	6	# aag	Lys(K)	3	# ---	Ter(.)	0
---	Ala(A)	8	# ---	Glu(E)	13	# ---	Lys(K)	10	# aca	Thr(T)	3
aga	Arg(R)	0	# gga	Gly(G)	6	# aug	Met(M)	2	# acc	Thr(T)	2
agg	Arg(R)	1	# ggc	Gly(G)	3	# ---	Met(M)	2	# acg	Thr(T)	1
cga	Arg(R)	1	# ggg	Gly(G)	3	# uuc	Phe(F)	4	# acu	Thr(T)	1
cgc	Arg(R)	5	# ggu	Gly(G)	4	# uuu	Phe(F)	5	# ---	Thr(T)	7
cgg	Arg(R)	0	# ---	Gly(G)	16	# ---	Phe(F)	9	# ugg	Trp(W)	2
cgu	Arg(R)	2	# cac	His(H)	0	# cca	Pro(P)	0	# ---	Trp(W)	2
---	Arg(R)	9	# cau	His(H)	2	# ccc	Pro(P)	0	# uac	Tyr(Y)	4
aac	Asn(N)	4	# ---	His(H)	2	# ccg	Pro(P)	1	# uau	Tyr(Y)	0
aau	Asn(N)	5	# aua	Ile(I)	0	# ccu	Pro(P)	9	# ---	Tyr(Y)	4
---	Asn(N)	9	# auc	Ile(I)	8	# ---	Pro(P)	10	# gua	Val(V)	0
gac	Asp(D)	3	# auu	Ile(I)	6	# agc	Ser(S)	1	# guc	Val(V)	0
gau	Asp(D)	14	# ---	Ile(I)	14	# agu	Ser(S)	1	# gug	Val(V)	8
---	Asp(D)	17	# cua	Leu(L)	1	# uca	Ser(S)	3	# guu	Val(V)	6
ugc	Cys(C)	1	# cuc	Leu(L)	3	# ucc	Ser(S)	1	# ---	Val(V)	14
ugu	Cys(C)	3	# cug	Leu(L)	6	# ucg	Ser(S)	1	# nnn	???(X)	0
---	Cys(C)	4	# cuu	Leu(L)	5	# ucu	Ser(S)	4	# TOTAL		181
caa	Gln(Q)	3	# uua	Leu(L)	0	# ---	Ser(S)	11	#		

[161] The optimized DNA was synthesized by MCLAB (South San Francisco) *in vitro* as a DNA fragment containing three restriction sites (*Nhe*I at 5' end of gene and *Xho*I and *BstE*II at the 3' end), a kexB cleaveage site and three glycine residues at N-terminal end and six histidine residues at C-terminal (SEQ I.D. NO:3). This optimized gene was cloned into a pCRII-TOPO vector. Following the procedures described in Example 1 above, the *Nhe*I to *BstE*II fragment was released from the plasmid by restriction digestion and the DNA fragment was purified on and extracted from an agarose gel and cloned into pSLGAMpR2 to create expression plasmid pSLGAMpR2-SBTI (Q107).

[162] The expression plasmid was transformed into dgr246ΔGAP:pyr2. The transformation and shake flask testing of transformants were as in Example 1. Thirty one transformants were assayed and SDS gel was used to check the level of protein expression. Broth from the top six transformants were assayed for trypsin inhibition activity.

Example 3
Expression of the Bowman-Birk Inhibitor and its Variants in *Aspergillus*

a. BBI fusion to glucoamylase with kexB site and with three glycine at N-terminal end and six histidine residues at C-terminal:

[163] Following procedures described in Example 2 above, the BBI-encoding DNA was optimized and used for this Example. The DNA was synthesized by MCLAB *in vitro* as a DNA fragment containing three restriction sites (*Nhe*I at 5' end of gene and *Xho*I and *BstE*II at the 3' end), a kexB cleavage site and three glycine residues at N-terminal and six histidine residues at C-terminal. (SEQ ID No:76). It was cloned into pCRII-TOPO vector Invitrogen. Following procedures described in Example 1 above, the *Nhe*I to *BstE*II fragment was released from the plasmid by restriction digestion and the DNA fragment was extracted from agarose gel and cloned into pSLGAMpR2 to create expression plasmid pSLGAMpR2-BBIkex+ (Q104). The expression plasmid was transformed into dgr246ΔGAP:pyr2. The transformation and shake flask testing of transformants were same as Example 1. Twenty-eight transformants were generated and twenty-five transformants were assayed in shake flask. The SDS gel was used to check the level of protein expression. Broth from the top transformants were assayed for trypsin or chymotrypsin inhibition activity.

b. BBI fusion to glucoamylase with six histidine residues at C-terminal:

[164] Following procedures described in Example 2 above, the BBI-encoding DNA was optimized and used for this Example. The DNA was synthesized by MCLAB *in vitro* as a DNA fragment containing three restriction sites (*Nhe*I at 5' end of gene and *Xho*I and *BstE*II

at the 3' end) and six histidine residues at C-terminal. (SEQ ID NO:42:
GCTAGCGACGATGAGAGCTCTAAGCCCTGTTGCGATCAGTGCGCGTGTACCAAATCGA
ACCCTCCGCAGTGTCGCTGCTCCGATATGCGTCTGAATTCCTGTCATAGCGCATGCAA
GAGCTGTATCTGCGCCCTGAGCTACCCCGCGCAGTGTTTCTGCGTCGACATCACGGAC
TTCTGCTACGAGCCGTGTAAGCCCAGCGAGGACGATAAGGAGAACCATCATCACCATC
ACCATTAGCTCGAGGGTGACC). It was cloned into pCRII-TOPO vector. Following procedures described in Example 1 above, the *Nhe*I to *BstE*II fragment was release from the plasmid by restriction digestion, purified and extracted from agarose gel, and cloned into pSLGAMpR2 to create expression plasmid pSLGAMpR2-BBIkex-(Q105). The expression plasmid was transformed into dgr246ΔGAP:pyr2. The transformation and shake flask testing of transformants were same as example 1. Thirty-eight transformants were generated and twenty-five transformants were assayed in shake flask. The SDS gel was used to check the level of protein expression. Broth from the top transformants were assayed for trypsin or chymotrypsin inhibition activity.

c. BBI fusion to glucoamylase with kexB site and three glycine residues at N-terminal end:

[165] The plasmid DNA, synthesized by MCLAB *in vitro* (SEQ ID NO:5) which was cloned into pCRII-TOPO vector, was used as DNA template for PCR amplification. Two primers were designed: 5' GGG CTA GCA ACG TCA TCT CCA AG 3' (SEQ ID NO:43) and 5' GGG GTC ACC TAG TTC TCC TTA TCG TCC TCG CTG 3' (SEQ ID NO:44). The DNA was amplified in the presence of the primers under the following conditions: The DNA was diluted 10 to 100 fold with Tris-EDTA buffer. Ten microliter of diluted DNA was added to the reaction mixture which contained 0.2 mM of each nucleotide (A, G. C and T), 1x reaction buffer, 0.5 to 0.6 microgram of primer 1 (SEQ ID NO:43) and primer 2 (SEQ ID NO:44) in a total of 100 microliter reaction in an eppendorf tube. After heating the mixture at 100°C for 5 minutes, 2.5 units of Taq DNA polymerase were added to the reaction mix. The PCR reaction was performed at 95°C for 1 minute, the primer was annealed to the template at 50°C for 1 minute and extension was done at 72°C for 1 minute. This cycle was repeated 30 times with an additional cycle of extension at 68°C for 7 minutes before stored at 4°C for further use. The PCR fragment detected by agarose gel was then cloned into the plasmid vector pCRII-TOPO (Invitrogen). The resulting PCR fragment contains identical sequence as SEQ ID NO:76, except the nucleotides encoding the six histidine residues and the *Xho*I restriction site were removed. Following procedures described in Example 1 above, the PCR fragment was digested with restriction enzymes *Nhe*I and *BstE*II. The digested DNA fragment was

precipitated by ethanol and cloned into pSLGAMpR2 to create expression plasmid pSLGAMpR2-BBI without histag (Q108). The expression plasmid was transformed into dgr246ΔGAP:pyr2. The transformation and shake flask testing of transformants were same as described in Example 1. Fifty-seven transformants were generated and twenty-five transformants were assayed in shake flask. The SDS gel was used to check the level of protein expression. Broth from the top transformants were assayed for trypsin or chymotrypsin inhibition activity.

d. BBI fusion to glucoamylase with kexB site:

[166] The plasmid DNA, synthesized by MCLAB in vitro (SEQ ID NO:1) which was cloned into pCRII-TOPO vector, was used as DNA template for PCR amplification. Two primers were designed: 5' GGG GTC ACC TAG TTC TCC TTA TCG TCC TCG CTG 3' (SEQ ID NO:44) and 5' GGG CTA GCA ACG TCA TCT CCA AGC GCG ACG ATG AGA GCT CTA AG 3' (SEQ ID NO:45). The resulting PCR fragment contains identical sequence as SEQ ID NO:76 (Figure 1C), except the nucleotides encoding the three glycine residues and six histidine residues and *Xho*I restriction site were removed. Following procedures described in Example 1 above, the PCR fragment was digested with restriction enzymes *Nhe*I and *BstE*II. The digested DNA fragment was precipitated by ethanol and cloned into pSLGAMpR2 to create expression plasmid pSLGAMpR2-BBI without 3G and histag (Q109). The expression plasmid was transformed into dgr246ΔGAP:pyr2. The transformation and shake flask testing of transformants were same as Example 1. One hundred and twenty-seven transformants were generated and forty-two transformants were assayed in shake flask. The SDS gel was used to check the level of protein expression. Broth from the top transformants were assayed for trypsin or chymostrypsin inhibition activity.

Example 4
Expression of the Bowman-Birk Inhibitor and its Variants (loop replacement by other binders) in *Aspergillus*

[167] Variant sequences were introduced into one or both loops of BBI using standard procedures known in the art. Variant sequences were determined by panning a commercially available phage peptide library PhD C7C (New England Biolabs, Beverly, MA) against target proteins or substrates for 3 rounds according to the manufacturers instructions, or using sequences with known activity. In the sequences provided below, the alterations introduced into the loop nucleotide sequence is indicated by lower case nucleotides.

a. BBI with a-VEGF (CK37281) in loop I

[168] The plasmid DNA, synthesized by MCLAB *in vitro* (SEQ ID NO:1) which was cloned into pCRII-TOPO vector, was used as DNA template for PCR amplification. Two primers were designed:

5' GTTGCGATCAGTGCGCGTGTtacaatctgtatggctggaccTGTCGCTGCT 3' (SEQ ID NO:46) and

5' CGCATATCGGAGCAGCGACAggtccagccatacagattgtaACACGCGCAC 3'. (SEQ ID NO:47)

to introduce a peptide sequence that binds to VEGF (denoted a-VEGF) to inhibit VEGF function. PCR was performed by heating mixture at 94°C for 2 min, then 30 cycles of reaction at 94°C for 30 second, 63°C for 30 second and 72°C for 30 second. After 30 cycles, the mixture was incubated at 72°C for 4 min before it was stored at 4 °C. The replacement binding loop was verified by DNA sequencing. The *Nhe*I to *BstE*II DNA fragment was released from plasmid by restriction digestion, purified and cloned into pSLGAMpR2 to create expression plasmid pSLGAMpR2-BBI (CK37281) in loop1 (Q117). The expression plasmid was transformed into dgr246ΔGAP:pyr2, The transformation and shake flask testing of transformants were same as in Example 1. More than thirty transformants were generated and forty-two transformants were assayed in shake flask. The SDS gel was used to check the level of protein expression.

b. BBI with a-VEGF (CK37281) peptide in loop II:

[169] For plasmid construction, obtaining fungal transformants and assaying fungal transformant in shake flasks, we following same procedures as described in example above, except the following two primers were used:

5' CATGCAAGAGCTGTATCTGCtacaatctgtatggctggaccCAGTGTTTCTG3' (SEQ ID NO:48)

5' GATGTCGACGCAGAAACACTGggtccagccatacagattgtaGCAGATACAG3'. (SEQ ID NO:49)

c. BBI with a-VEGF (CK37281) peptide in loop I and II:

[170] For plasmid construction, obtaining fungal transformants and assaying fungal transformant in shake flasks, we following same procedures, except the following four primers were used:

5' GTTGCGATCAGTGCGCGTGTtacaatctgtatggctggaccTGTCGCTGCT 3' (SEQ ID NO:46)

5' CGCATATCGGAGCAGCGACAggtccagccatacagattgtaACACGCGCAC 3' (SEQ ID NO:47)

5' CATGCAAGAGCTGTATCTGCtacaatctgtatggctggaccCAGTGTTTCTG3' (SEQ ID NO:48)
5' GATGTCGACGCAGAAACACTGggtccagccatacagattgtaGCAGATACAG3'. (SEQ ID NO:49)

d. BBI with a-complement protein c2 peptide in loop I:

[171] For plasmid construction, obtaining fungal transformants and assaying fungal transformant in shake flasks, we following same procedures, except the following two primers were used to introduce a peptide sequence that binds to c2 (denoted a-c2) to inhibit c2 function:
5'GCGATCAGTGCAGCTGTagctgcggcaggaagatccccatccagtgcTGTCGCTGCTCCGATATGC GTC3' (SEQ ID NO:50)
5'GAGCAGCGACAgcactggatggggatcttcctgccgcagctACAGCTGCACTGATCGCAACAGGGC TTA3' (SEQ ID NO:51)

e. BBI with a-complement protein c3 peptide in loop I:

[172] For plasmid construction, obtaining fungal transformants and assaying fungal transformant in shake flasks, we following same procedures, except the following two primers were used to introduce a peptide sequence that binds to c3 (denoted a-c3) to inhibit c3 function:
5' GCGATCAGTGCGGCTGTgccaggagcaacctcgacgagTGTCGCTGCTCCGATATGCGTC 3' (SEQ ID NO:52)
5' GAGCAGCGACActcgtcgaggttgctcctggcACAGCCGCACTGATCGCAACAGGGCTTA 3' (SEQ ID NO:53)

f. BBI with a-complement protein c4 peptide in loop I:

[173] For plasmid construction, obtaining fungal transformants and assaying fungal transformant in shake flasks, we following same procedures, except the following two primers were used to introduce a peptide sequence that binds to c4 (denoted a-c4) to inhibit c4 function:
5' GCGATCAGTGCGCGTGTcagagggccctccccatcctcTGTCGCTGCTCCGATATGCGTC 3' (SEQ ID NO:55)
5' GAGCAGCGACAgaggatggggagggccctctgACACGCGCACTGATCGCAACAGGGCTTA 3' (SEQ ID NO:56)

g. BBI with a-complement protein c5 peptide in loop I:

[174] For plasmid construction, obtaining fungal transformants and assaying fungal transformant in shake flasks, we following same procedures, except the following two primers were used to introduce a peptide sequence that binds to c5 (denoted a-c5) to inhibit c5 function:

5' GCGATCAGTGCCAGTGTggcaggctccacatgaagaccTGTCGCTGCTCCGATATGCGTC 3' (SEQ ID NO:57)

5' GAGCAGCGACAggtcttcatgtggagcctgccACACTGGCACTGATCGCAACAGGGCTTAGA 3' (SEQ ID NO:58)

h. BBI with a-human complement Factor B peptide in loop I:

[175] For plasmid construction, obtaining fungal transformants and assaying fungal transformant in shake flasks, we following same procedures, except the following two primers were used to introduce a peptide sequence that binds to Factor B (denoted a-Factor B) to inhibit Factor B function:

5' GCGATCAGTGCCAGTGTaagaggaagatcgtcctcgacTGTCGCTGCTCCGATATGCGTC 3' (SEQ ID NO:59)

5' GAGCAGCGACAgtcgaggacgatcttcctcttACACTGGCACTGATCGCAACAGGGCTTAGA 3' (SEQ ID NO:60)

i. BBI with a-Membrane Metalloprotease 2 (MMP2) peptide in loop I:

[176] For plasmid construction, obtaining fungal transformants and assaying fungal transformant in shake flasks, we following same procedures, except the following two primers were used to introduce a peptide sequence that binds to MMP2 (denoted a- MMP2) to inhibit MMP2 function:

5' CAGTGCGCGTGTgccgccatgttcggccccgccTGTCGCTGCTCCGATATGCGTC 3' (SEQ ID NO:61)

5' GAGCAGCGACAggcggggccgaacatggcggcACACGCGCACTGATCGCAACAG 3' (SEQ ID NO:62)

j. BBI with a-Membrane Metalloprotease 12 (MMP12) peptide in loop I:

[177] For plasmid construction, obtaining fungal transformants and assaying fungal transformant in shake flasks, we following same procedures, except the following two

primers were used to introduce a peptide sequence that binds to MMP12 (denoted a-MMP12) to inhibit MMP12 function:
5' CAGTGCGCGTGTggcgccctcggcctcttcggcTGTCGCTGCTCCGATATGCGTC 3' (SEQ ID NO:63)
5' GAGCAGCGACAgccgaagaggccgagggcgccACACGCGCACTGATCGCAACAG 3' (SEQ ID NO:64)

k. BBI with cotton binding peptide 2314 in loop I:

[178] For plasmid construction, obtaining fungal transformants and assaying fungal transformant in shake flasks, we following same procedures, except the following two primers were used to introduce a peptide sequence that binds to cotton:
5' GTTGCGATCAGTGCGCGTGTgagcccctgatccaccagcgcTGTCGCTGCT 3' (SEQ ID NO:65)
5' CGCATATCGGAGCAGCGACAgcgctggtggatcaggggctcACACGCGCAC 3' (SEQ ID NO:66)

l. BBI with cotton binding peptide 2317 in loop I:

[179] For plasmid construction, obtaining fungal transformants and assaying fungal transformant in shake flasks, we following same procedures, except the following two primers were used to introduce a peptide sequence that binds to cotton:
5' GTTGCGATCAGTGCGCGTGTagcgccttccgcggccccaccTGTCGCTGCT3' (SEQ ID NO:67)
5' CGCATATCGGAGCAGCGACAggtggggccgcggaaggcgctACACGCGCAC 3' (SEQ ID NO:68)

m. BBI with compstatin loop in loop I:

[180] For plasmid construction, obtaining fungal transformants and assaying fungal transformant in shake flasks, we following same procedures, except the following two primers were used to introduce the compstatin peptide sequence:
5' GTTGCGATCAGTGCGCGTGTgttgttcaggactggggccaccaccgcTGTCGCTGCT (SEQ ID NO:69)
5' CGCATATCGGAGCAGCGACAgcggtggtggccccagtcctgaacaacACACGCGCAC (SEQ ID NO:70)

[181] In this case, the 7 amino acids from the BBI Trypsin binding loop was replaced by 9 amino acids from compstatin binding loops.

n. BBI with compstatin loop in loop II:

[182] For plasmid construction, obtaining fungal transformants and assaying fungal transformant in shake flasks, we following same procedures, except the following two primers were used to introduce the compstatin peptide sequence:

5' CATGCAAGAGCTGTATCTGCgttgttcaggactggggccaccaccgcTGTTTCTGCG (SEQ ID NO:71)

5' GTGATGTCGACGCAGAAACAgcggtggtggccccagtcctgaacaacGCAGATACAG (SEQ ID NO:72)

[183] In this case, the 7 amino acids from the BBI Trypsin binding loop was replaced by 9 amino acids from compstatin binding loops.

Example 5

Expression of the Bowman-Birk Inhibitor and its Variants in *Trichoderma reesei*

[184] Following procedures described in Example 2 above, the BBI-encoding DNA was optimized and used for this Example. Two primers were designed to amplify the DNA fragment using plasmid pSLGAMpR2-BBI or pSLGAMpR2-BBI with a-VEGF (CK37281) peptide in loop I and II as templates:

5' GGA CTA GTA AGC GCG ACG ATG AGA GCT CT 3' (SEQ ID NO:73)

5' AAG GCG CGC CTA GTT CTC CTT ATC GTC CT 3' (SEQ ID NO:74)

A third primer was also used to create a PCR fragment which contains three glycine residues at the N-terminal of the BBI protein when used in conjunction with primer #2 (SEQ ID NO:74) above.

5' GGA CTA GTA AGC GCG GCG GTG GCG ACG ATG AGA GCT CT 3' (SEQ ID NO:75).

[185] Following the same procedures described in Example 2 above, the BBI-encoding DNA was optimized and used for this Example, the PCR fragment was cut with restriction enzyme *Spe*I and *Asc*I and ligated to the Trichoderma expression plasmid, pTrex4 (Figure 8) which is a modified version of pTREX2 (see Figure 9), which in turn is a modified version of pTEX, see PCT Publication No. WO 96/23928 for a complete description of the preparation of the pTEX vector, herein incorporated by reference, which contains a CBHI promoter and terminator for gene expression and a Trichoderma *pyr4* gene as a selection marker for transformants, to create an expression plasmid. In the pTrex4 plasmid, the BBI gene was fused to the C-terminus of the CBH I core and linker from *T. reesei*. The *amdS* gene from *A. nidulans* was used as the selection marker during fungal transformation. The expression plasmid was transformed into *Trichoderma reesei*. Stable transformants were isolated on

Trichoderma minimal plates with acetamide as the nitrogen source. The transformants were grown on the amd minus plate which contains 1 ml/l 1000X salts, 20g/l Noble Agar, 1.68g/l CsCl, 20g/l Glucose, 15g/l KH2PO4, 0.6g/l MgSO4*7H2O, 0.6g/l CaCl2*2H2O and 0.6g/l Acetamide. The final pH was adjusted to 4.5. The 1000x salts contains 5g/l FeSO4, 1.6g/l MnSO4, 1.4g/l ZnSO4 and 1g/l CoCl2. It was filter sterilized. After three days incubation at 28°C, the transformants were transferred to the fresh amd minus plates and grown for another three days at 28°C.

[186] The transformants were then inoculated into *T. reesei* proflo medium (50 ml for each transformant) in 250-ml shake flasks. *T. reesei* proflo medium contains 30g/l Alpha-lactose, 6.5g/l (NH4)2SO4, 2g/l KH2PO4, 0.3g/l MgSO4*7H2O, 0.2g/l CaCl2, 1ml/l 1000x TRI Trace Salts, 2ml/l 10% Tween 80, 22.5g/l Proflo and 0.72g/l CaCO3. The 1000x TRI Trace Salts contains 5g/l FeSO4 * 7H2O, 1.6g/l MnSO4 * H2O and 1.4g/l ZnSO4 * 7H2O. After growing at 30°C for 2 days, 4 ml of culture was transferred into defined medium which contains 5g/l (NH4)2SO4, 33g/l PIPPS buffer, 9g/l CASAMINO ACIDS, 4.5g/l KH2PO4, 1g/l CACL2, 1g/l MgSO4*7H2O, 5ml/l MAZU and 2.5ml/l 400X T.reesei TRACE. Its pH was adjusted to 5.5 and 40ml/1 40% lactose was added after sterilization. The 400X *T.reesei* TRACE contains 175g/l Citric Acid (anhydrous), 200g/l FeSO4 * 7H2O, 16g/l ZnSO4 * 7H2O, 3.2g/l CuSO4 * 5H2O, 1.4g/l MnSO4 * H2O and 0.8g/l H3BO3 (Boric Acid).

[187] About 40 transformants were generated on the plates and 20 were assayed in shake flasks. The supernatant of the culture was used for SDS-PAGE analysis and assayed for trypsin or chymotrypsin inhibitory activity. Western blot also showed the presence of both fusion (CbhI-BBI) and BBI alone.

Example 6

Co-Expression of the Bowman-Birk Inhibitor and Secretory Chaperones in *Aspergillus*

[188] The following example details how secretion can be enhanced. STI protein contains two disulfide bonds and BBI contains 7 disulfide bonds in their tertiary structures and these disulfide bonds are important for their function. It is known that folding of protein with disulfide bonds require Protein Disulfide Isomerase (PDI) or other chaprones in ER.

[189] Enhancement of STI or BBI expression was investigated by co-transformation of two plasmids or by sequential transformation of two plasmids, one contains STI or BBI expression cassette and the other one contains the PDI genes or chaperone genes. First, we co-transform plasmid pSLGAMpR2-BBI without 3G and histag (Q109) with plasmid Q51 which contains 4.6 kb genomic DNA covering region of the pdiA gene from *Aspergillus niger* in vector pUC219 into same strain (dgr246ΔGAP:pyr2). Fifty-one transformants were

obtained and forty-seven transformants were screened in shake flasks. Transformant #14 was selected because it produced the highest amount of BBI protein based on SDS gel data. The expression level of BBI protein is higher in the co-transformed stain than the strain containing only plasmid pSLGAMpR2-BBI without 3G and histag (Q109). Figure 7 illustrates the enhanced BBI expression. This strain was also spore purified and tested again in shake flask.

[190] Following procedures described above, we also decide to co-transform plasmid pSLGAMpR2-BBI without histag (Q108) with plasmid Q51 containing pdiA gene (same as above) into same strain (dgr246ΔGAP:pyr2). Thirty-four transformants were screened in shake flasks. One transformant was selected for its ability to produce BBI protein at the highest level based on the SDS gel date. The expression level of BBI protein is higher than the strain containing only plasmid pSLGAMpR2- BBI without histag (Q108).

[191] Following procedures described above, we also decide to co-transform plasmid pSLGAMpR2-BBI without 3G and histag (Q109) with plasmid Q124 which contains 1623 bp genomic DNA covering region of the prpA gene from *Aspergillus niger* in vector pUC219 into same strain (dgr246ΔGAP:pyr2). Twenty-eight transformants were screened in shake flasks. One transformant was selected for its ability to produced the highest amount of BBI protein based on the SDS gel date. The expression level of BBI protein is higher in the co-transformed stain than the strain containing only plasmid pSLGAMpR2- BBI without 3G and histag (Q109). Figure 7 illustrates the enhancement (lane 15 vs lane 3). This strain was spore purified and tested again in shake flask.

Example 7
Recombinant Protease Inhibitor Variants Retain Activity

[192] STI, BBI and variants thereof produced using the methods described above were tested for activity, e.g., inhibition of protease activity.

a. Protease inhibition

[193] 950 μl of Tris-buffered saline + 0.02% Tween 20 is combined with 20 μl protease (100μg/ml in 1mM HCl (bovine trypsin or chymotrypsin)) and 20 μl sample. The solution is mixed and incubated for 30 min. at room temperature. 10 μl substrate (for trypsin: succinyl-ala-ala-pro-arg-paranitroanilide, 10 mg/ml in DMSO; for chymotrypsin: succinyl ala-ala-pro-phe-paranitroanilide, 10 mg/ml in DMSO) is added and the solution mixed. Absorbance is monitored at 405nm and the rate determined (A_{405}/min). The fraction of protease activity inhibited is determined by comparison with a control sample blank and calculated according to the following equation:

$$\left(\frac{A_{405}/\min(sample)}{A_{405}/\min(blank)}\right)*100\mu g/ml(protease)*\left(\frac{MWinhibitor}{MWprotease}\right)=[inhibitor]\mu g/ml$$

b. Inhibition of HUVEC proliferation by aVEGF peptides.

[194] HUVE cells (Cambrex, East Rutherford, NJ) were passaged 1-5 times and maintained according to manufacturers instructions. HUVEC growth was stimulated by 0.03 to 20 ng/ml VEGF with the highest proliferation at 10 ng/ml $VEGF_{165}$ (R&D systems); this concentration was used in subsequent experiments. A series of a-VEGF peptides (see Example 4) from 0.00052 μM to 25 μM and an anti-VEGF MAb control (R&D Systems) were mixed with 10 ng/mL VEGF prior to addition to HUVECs seeded in triplicate in 96-well plates. Cell proliferation was measured by ^{3}H-thymidine incorporation. Significant inhibition was observed (data not shown).

[195] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

ABSTRACT OF THE DISCLOSURE

[196] Described herein are protease inhibitors, variants thereof and methods for their production.

Figure 1A: Codon optimized BBI (SEQ ID NO:1)

```
GCTAGCAACG TCATCTCCAA GCGCGACGAT GAGAGCTCTA AGCCCTGTTG        50
NheI

CGATCAGTGC GCGTGTACCA AATCGAACCC TCCGCAGTGT CGCTGCTCCG       100

ATATGCGTCT GAATTCCTGT CATAGCGCAT GCAAGAGCTG TATCTGCGCC       150

CTGAGCTACC CCGCGCAGTG TTTCTGCGTC GACATCACGG ACTTCTGCTA       200

CGAGCCGTGT AAGCCCAGCG AGGACGATAA GGAGAACTAG CTCGAGGGTG       250
                                        ***  XhoI ####
ACC                                                         253
###
```

Figure 1B: Codon optimized BBI with three glycine (SEQ ID NO:5)

```
GCTAGCAACG TCATCTCCAA GCGC**GGCGGT GGC**GACGATG AGAGCTCTAA    50
NheI

GCCCTGTTGC GATCAGTGCG CGTGTACCAA ATCGAACCCT CCGCAGTGTC       100

GCTGCTCCGA TATGCGTCTG AATTCCTGTC ATAGCGCATG CAAGAGCTGT       150

ATCTGCGCCC TGAGCTACCC CGCGCAGTGT TTCTGCGTCG ACATCACGGA       200

CTTCTGCTAC GAGCCGTGTA AGCCCAGCGA GGACGATAAG GAGAACTAGC       250
                                                    ***

TCGAGGGTGA CC                                               262
XhoI ##### ##
```

Figure 1C: Codon optimized BBI with three glycine at N-terminal end and six histidine residues at C-terminal end (SEQ ID NO:76)

```
GCTAGCAACG TCATCTCCAA GCGC**GGCGGT GGC**GACGATG AGAGCTCTAA    50
NheI

GCCCTGTTGC GATCAGTGCG CGTGTACCAA ATCGAACCCT CCGCAGTGTC       100

GCTGCTCCGA TATGCGTCTG AATTCCTGTC ATAGCGCATG CAAGAGCTGT       150

ATCTGCGCCC TGAGCTACCC CGCGCAGTGT TTCTGCGTCG ACATCACGGA       200

CTTCTGCTAC GAGCCGTGTA AGCCCAGCGA GGACGATAAG GAGAACCACC       250
                                                    ••••

ATCACCATCA CCACTAGCTC GAGGGTGACC                             280
•••••••••• ••••***  XhoI #######
```

Figure 2: Codon Optimized STI (SEQ ID NO:3)

```
GCTAGCAACG TCATCTCCAA GCGCGGCGGT GGCGATTTCG TGCTCGATAA          50
NheI

TGAAGGCAAC CCTCTTGAAA ATGGTGGCAC ATACTACATC CTGTCAGACA         100

TCACAGCATT TGGTGGAATC CGCGCAGCCC CTACGGGAAA TGAACGCTGC         150

CCTCTCACTG TGGTGCAATC TCGCAATGAG CTCGACAAAG GGATTGGAAC         200

AATCATCTCG TCCCCTTACC GAATCCGTTT TATCGCCGAA GGCCATCCTC         250

TGAGCCTTAA GTTCGATTCA TTTGCAGTTA TCATGCTGTG TGTTGGAATT         300

CCTACCGAGT GGTCTGTTGT GGAGGATCTA CCTGAAGGAC CTGCTGTTAA         350

AATTGGTGAG AACAAAGATG CAATGGATGG TTGGTTTCGC CTTGAGCGCG         400

TTTCTGATGA TGAATTCAAT AACTACAAGC TTGTGTTCTG TCCTCAGCAA         450

GCTGAGGATG ACAAATGTGG GGATATTGGG ATTAGTATTG ATCATGATGA         500

TGGAACCAGG CGTCTGGTGG TGTCTAAGAA CAAACCGCTG GTGGTTCAGT         550

TTCAAAAACT TGATAAAGAA TCACTGCACC ATCACCATCA CCACTAGCTC         600
                                  ••••••••••••••••••••••***Xho

GAGGGTGACC                                                    610
I  #######
```

Figure 3A: BBI amino acid sequence (SEQ ID NO:7)

DDESSKPCCD QCACTKSNPP QCRCSDMRLN SCHSACKSCI C**ALSYPAQ**CF 50
CVDITDFCYE PCKPSEDDKE N 71

Figure 3B: BBI with three glycine residues at N-terminal end (SEQ ID NO:8):

GGGDDESSKP CCDQCACTKS NPPQCRCSDM RLNSCHSACK SCIC**ALSYPA** 50
QCFCVDITDF CYEPCKPSED DKEN 74

Figure 3C: BBI with three glycine residues at N-terminal end and six histidine residues at C-terminal end (SEQ ID NO:9):

GGGDDESSKP CCDQCACTKS NPPQCRCSDM RLNSCHSACK SCIC**ALSYPA** 50
QCFCVDITDF CYEPCKPSED DKENHHHHHH 80

Figure 4A: STI amino acid sequence (with glycine & His tag) (SEQ ID NO:10)

GGGDFVLDNE GNPLENGGTY YILSDITAFG GIRAAPTGNE RCPLTVVQSR 50
***

NELDKGIGTI ISSPYRIRFI AEGHPLSLKF DSFAVIMLCV GIPTEWSVVE 100

DLPEGPAVKI GENKDAMDGW FRLERVSDDE FNNYKLVFC**P QQAEDDK**CGD 150

IGISIDHDDG TRRLVVSKNK PLVVQFQKLD KESLHHHHHH 190

Figure 4B: STI amino acid sequence (without His tag) (SEQ ID NO:11)

GGGDFVLDNE GNPLENGGTY YILSDITAFG GIRAAPTGNE RCPLTVVQSR 50
***

NELDKGIGTI ISSPYRIRFI AEGHPLSLKF DSFAVIMLCV GIPTEWSVVE 100

DLPEGPAVKI GENKDAMDGW FRLERVSDDE FNNYKLVFC**P QQAEDDK**CGD 150

IGISIDHDDG TRRLVVSKNK PLVVQFQKLD KESL 184

Figure 4C: STI amino acid sequence (SEQ ID NO:12)

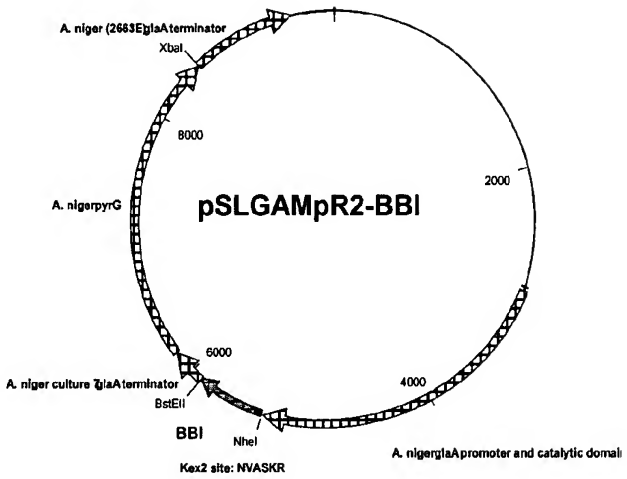
DFVLDNEGNP LENGGTYYIL SDITAFGGIR AAPTGNERCP LTVVQSRNEL 50

DKGIGTIISS PYRIRFIAEG HPLSLKFDSF AVIMLCVGIP TEWSVVEDLP 100

EGPAVKIGEN KDAMDGWFRL ERVSDDEFNN YKLVFC**PQQA EDDK**CGDIGI 150

SIDHDDGTRR LVVSKNKPLV VQFQKLDKES L 181

Figure 5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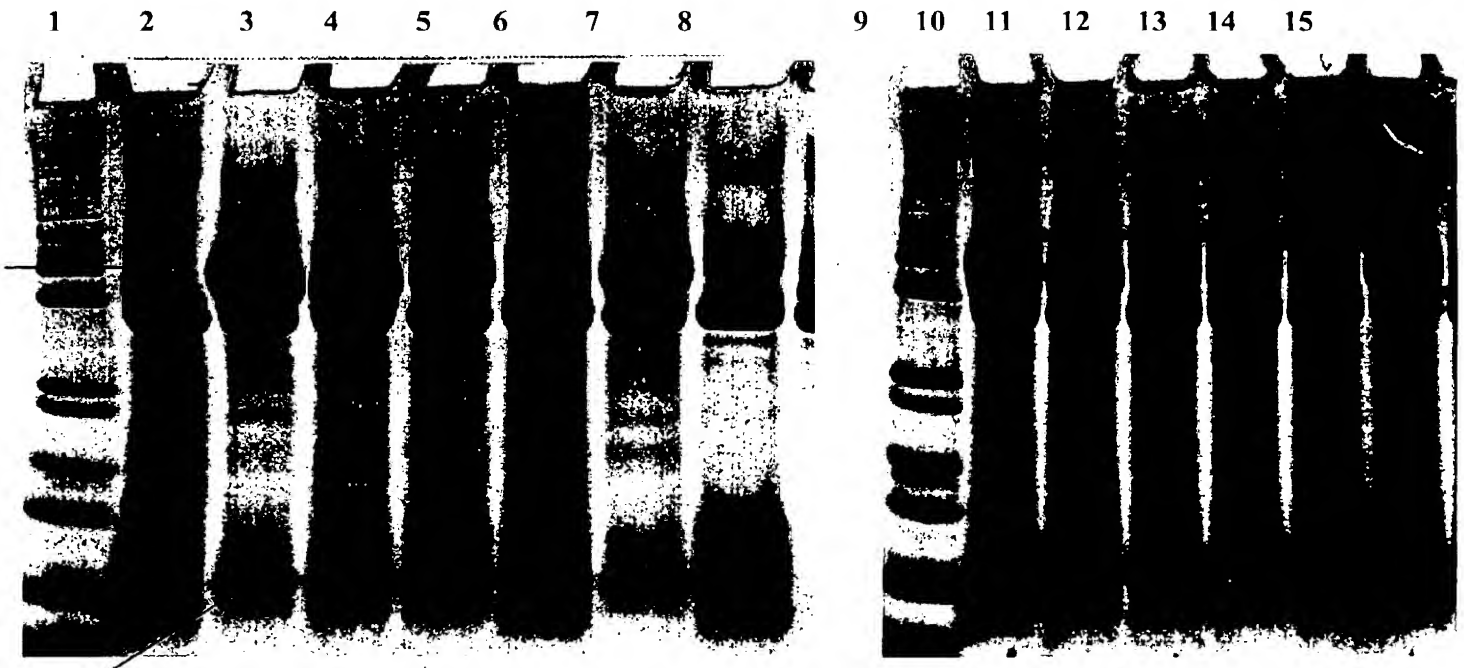
6000
4000
A. niger culture EglaAterminator
BstEII
BBI
NheI
A. nigerglaApromoter and catalytic domai
Kex2 site: NVASKR

Figure 6:

Wild-type	DDESSKPCCD QCACTKSNPP QCRCSDMRLN SCHSACKSCI CALSYPAQCF CVDITDFCYE PCKPSEDDKE N	(SEQ ID NO:7)
VEGF	DDESSKPCCD QCACYNLYGW TCRCSDMRLN SCHSACKSCI CALSYPAQCF CVDITDFCYE PCKPSEDDKE N	(SEQ ID NO:15)
VEGF	DDESSKPCCD QCACTKSNPP QCRCSDMRLN SCHSACKSCI CYNLYGWTCF CVDITDFCYE PCKPSEDDKE N	(SEQ ID NO:16)
VEGF	DDESSKPCCD QCACYNLYGW TCRCSDMRLN SCHSACKSCI CYNLYGWTCF CVDITDFCYE PCKPSEDDKE N	(SEQ ID NO:17)
C2	DDESSKPCCD QCSCGRKIPI QCRCSDMRLN SCHSACKSCI CALSYPAQCF CVDITDFCYE PCKPSEDDKE N	(SEQ ID NO:18)
C3	DDESSKPCCD QCGCARSNLD ECRCSDMRLN SCHSACKSCI CALSYPAQCF CVDITDFCYE PCKPSEDDKE N	(SEQ ID NO:19)
C4	DDESSKPCCD QCGCQRALPI LCRCSDMRLN SCHSACKSCI CALSYPAQCF CVDITDFCYE PCKPSEDDKE N	(SEQ ID NO:20)
C5	DDESSKPCCD QCQCGRLHMK TCRCSDMRLN SCHSACKSCI CALSYPAQCF CVDITDFCYE PCKPSEDDKE N	(SEQ ID NO:21)
Factor B	DDESSKPCCD QCQCKRKIVL DCRCSDMRLN SCHSACKSCI CALSYPAQCF CVDITDFCYE PCKPSEDDKE N	(SEQ ID NO:22)
MMP-2	DDESSKPCCD QCACAAMFGP ACRCSDMRLN SCHSACKSCI CALSYPAQCF CVDITDFCYE PCKPSEDDKE N	(SEQ ID NO:23)
MMP-12	DDESSKPCCD QCACGALGLF GCRCSDMRLN SCHSACKSCI CALSYPAQCF CVDITDFCYE PCKPSEDDKE N	(SEQ ID NO:24)
CBP1	DDESSKPCCD QCACEPLIHQ RCRCSDMRLN SCHSACKSCI CALSYPAQCF CVDITDFCYE PCKPSEDDKE N	(SEQ ID NO:25)
CBP2	DDESSKPCCD QCACSAFRGP TCRCSDMRLN SCHSACKSCI CALSYPAQCF CVDITDFCYE PCKPSEDDKE N	(SEQ ID NO:26)
C-statin	DDESSKPCCD QCACVVQDWG HHRCRCSDMR LNSCHSACKS CICALSYPAQ CFCVDITDFC YEPCKPSEDD KEN	(SEQ ID NO:27)
C-statin	DDESSKPCCD QCACTKSNPP QCRCSDMRLN SCHSACKSCI CVVQDWGHHR CFCVDITDFC YEPCKPSEDD KEN	(SEQ ID NO:28)
C-statin	DDESSKPCCD QCACVVQDWG HHRCRCSDMR LNSCHSACKS CVVQDWGHHR CFCVDITDFC YEPCKPSEDD KEN	(SEQ ID NO:29)

VEGF	C**YNLYGWT**C	(SEQ ID NO:30)
C2	C**SCGRKIPIQ**C	(SEQ ID NO:31)
C3	C**GCARSNLDE**C	(SEQ ID NO:32)
C4	C**GCQRALPIL**C	(SEQ ID NO:33)
C5	C**QCGRLHMKT**C	(SEQ ID NO:34)
Factor B	C**QCKRKIVLD**C	(SEQ ID NO:35)
MMP-2	C**AAMFGPA**C	(SEQ ID NO:36)
MMP-12	C**GALGLFG**C	(SEQ ID NO:37)
cotton binding peptide	C**EPLIHQR**C	(SEQ ID NO:38)
cotton binding peptide	C**SAFRGPT**C	(SEQ ID NO:39)
Compstatin	C**VVQDWGHHR**C	(SEQ ID NO:40)

Summary of best transformants

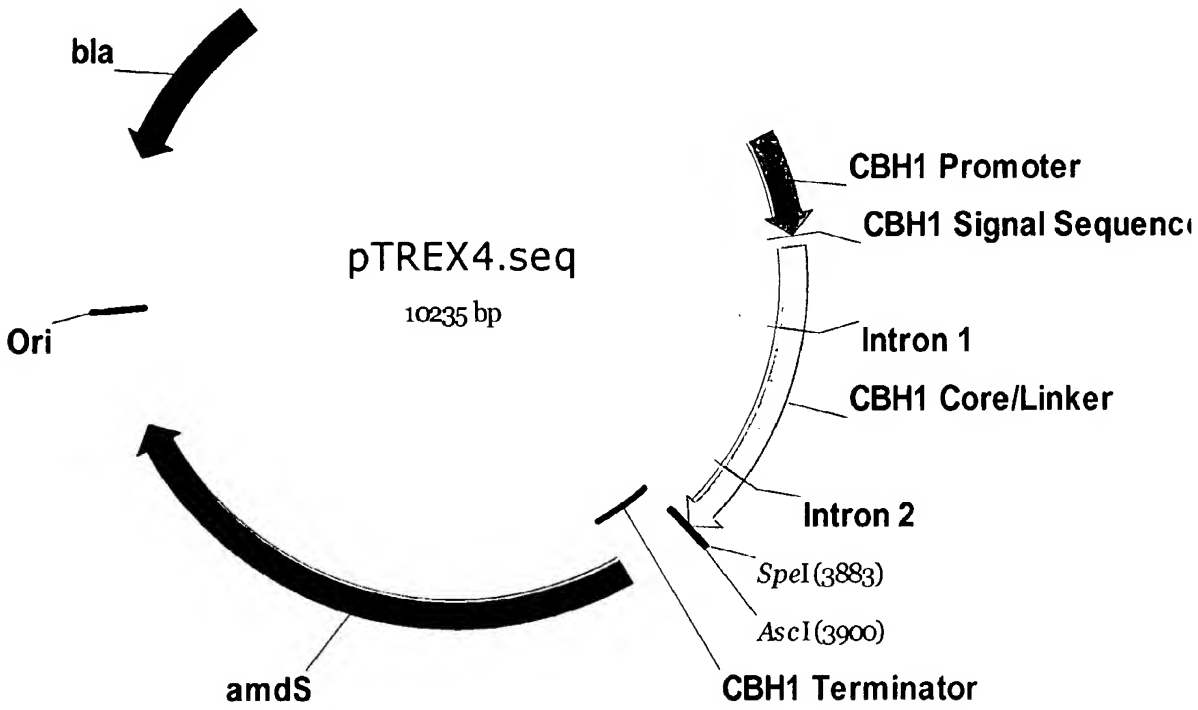


Lane 1: marker
Lane 2 untransformed parental strain
Lane 3: transformant #Q109-7 (BBI)
Lane 4: transformant #Q109/Q51-14 (BBI+pdiA)

Lane 11: transformant #Q109/Q51-14d (BBI+pdiA, spore purified clone)
Lane 15: transformant #Q109/Q124-22 (BBI+prpA)

Figure 7

Figure 8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Ori
CBH1 Promoter
CBH1 Signal Sequenc
Intron 1
CBH1 Core/Linker
Intron 2
SpeI (3883)
AscI (3900)
amdS
CBH1 Terminator

FIGURE 9A: pTEX2

```
AAGCTTAAGG TGCACGGCCC ACGTGGCCAC TAGTACTTCT CGAGCTCTGT         50
ACATGTCCGG TCGCGACGTA CGCGTATCGA TGGCGCCAGC TGCAGGCGGC        100
CGCCTGCAGC CACTTGCAGT CCCGTGGAAT TCTCACGGTG AATGTAGGCC        150
TTTTGTAGGG TAGGAATTGT CACTCAAGCA CCCCCAACCT CCATTACGCC        200
TCCCCCATAG AGTTCCCAAT CAGTGAGTCA TGGCACTGTT CTCAAATAGA        250
TTGGGGAGAA GTTGACTTCC GCCCAGAGCT GAAGGTCGCA CAACCGCATG        300
ATATAGGGTC GGCAACGGCA AAAAAGCACG TGGCTCACCG AAAAGCAAGA        350
TGTTTGCGAT CTAACATCCA GGAACCTGGA TACATCCATC ATCACGCACG        400
ACCACTTTGA TCTGCTGGTA AACTCGTATT CGCCCTAAAC CGAAGTGCGT        450
GGTAAATCTA CACGTGGGCC CCTTTCGGTA TACTGCGTGT GTCTTCTCTA        500
GGTGCCATTC TTTTCCCTTC CTCTAGTGTT GAATTGTTTG TGTTGGAGTC        550
CGAGCTGTAA CTACCTCTGA ATCTCTGGAG AATGGTGGAC TAACGACTAC        600
CGTGCACCTG CATCATGTAT ATAATAGTGA TCCTGAGAAG GGGGGTTTGG        650
AGCAATGTGG GACTTTGATG GTCATCAAAC AAAGAACGAA GACGCCTCTT        700
TTGCAAAGTT TTGTTTCGGC TACGGTGAAG AACTGGATAC TTGTTGTGTC        750
TTCTGTGTAT TTTTGTGGCA ACAAGAGGCC AGAGACAATC TATTCAAACA        800
CCAAGCTTGC TCTTTTGAGC TACAAGAACC TGTGGGGTAT ATATCTAGAG        850
TTGTGAAGTC GGTAATCCCG CTGTATAGTA ATACGAGTCG CATCTAAATA        900
CTCCGAAGCT GCTGCGAACC CGGAGAATCG AGATGTGCTG GAAAGCTTCT        950
AGCGAGCGGC TAAATTAGCA TGAAAGGCTA TGAGAAATTC TGGAGACGGC       1000
TTGTTGAATC ATGGCGTTCC ATTCTTCGAC AAGCAAAGCG TTCCGTCGCA       1050
GTAGCAGGCA CTCATTCCCG AAAAAACTCG GAGATTCCTA AGTAGCGATG       1100
GAACCGGAAT AATATAATAG GCAATACATT GAGTTGCCTC GACGGTTGCA       1150
ATGCAGGGGT ACTGAGCTTG GACATAACTG TTCCGTACCC CACCTCTTCT       1200
CAACCTTTGG CGTTTCCCTG ATTCAGCGTA CCCGTACAAG TCGTAATCAC       1250
TATTAACCCA GACTGACCGG ACGTGTTTTG CCCTTCATTT GGAGAAATAA       1300
TGTCATTGCG ATGTGTAATT TGCCTGCTTG ACCGACTGGG GCTGTTCGAA       1350
GCCCGAATGT AGGATTGTTA TCCGAACTCT GCTCGTAGAG GCATGTTGTG       1400
AATCTGTGTC GGGCAGGACA CGCCTCGAAG GTTCACGGCA AGGGAAACCA       1450
CCGATAGCAG TGTCTAGTAG CAACCTGTAA AGCCGCAATG CAGCATCACT       1500
GGAAAATACA AACCAATGGC TAAAAGTACA TAAGTTAATG CCTAAAGAAG       1550
TCATATACCA GCGGCTAATA ATTGTACAAT CAAGTGGCTA AACGTACCGT       1600
AATTTGCCAA CGGCTTGTGG GGTTGCAGAA GCAACGGCAA AGCCCCACTT       1650
CCCCACGTTT GTTTCTTCAC TCAGTCCAAT CTCAGCTGGT GATCCCCCAA       1700
TTGGGTCGCT TGTTTGTTCC GGTGAAGTGA AAGAAGACAG AGGTAAGAAT       1750
GTCTGACTCG GAGCGTTTTG CATACAACCA AGGGCAGTGA TGGAAGACAG       1800
TGAAATGTTG ACATTCAAGG AGTATTTAGC CAGGGATGCT TGAGTGTATC       1850
GTGTAAGGAG GTTTGTCTGC CGATACGACG AATACTGTAT AGTCACTTCT       1900
GATGAAGTGG TCCATATTGA AATGTAAGTC GGCACTGAAC AGGCAAAAGA       1950
TTGAGTTGAA ACTGCCTAAG ATCTCGGGCC CTCGGGCCTT CGGCCTTTGG       2000
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GCGAATGTGT ATATATAAAG GTTCGAGGTC CGTGCCTCCC TCATGCTCTC       2250
CCCATCTACT CATCAACTCA GATCCTCCAG GAGACTTGTA CACCATCTTT       2300
TGAGGCACAG AAACCCAATA GTCAACCGCG GTTTAGGCGC GCCAGCTCCG       2350
TGCGAAAGCC TGACGCACCG GTAGATTCTT GGTGAGCCCG TATCATGACG       2400
GCGGCGGGAG CTACATGGCC CCGGGTGATT TATTTTTTTT GTATCTACTT       2450
```

FIGURE 9B: pTEX2

```
CTGACCCTTT TCAAATATAC GGTCAACTCA TCTTTCACTG GAGATGCGGC       2500
CTGCTTGGTA TTGCGATGTT GTCAGCTTGG CAAATTGTGG CTTTCGAAAA       2550
CACAAAACGA TTCCTTAGTA GCCATGCATT TTAAGATAAC GGAATAGAAG       2600
AAAGAGGAAA TTAAAAAAAA AAAAAAAACA AACATCCCGT TCATAACCCG       2650
TAGAATCGCC GCTCTTCGTG TATCCCAGTA CCAGTTTAAA CGGATCTCAA       2700
GCTTGCATGC AAAGATACAC ATCAATCGCA GCTGGGGTAC AATCATCCAT       2750
CATCCCAACT GGTACGTCAT AACAAAAATC GACAAGATGG AAAAAGAGGT       2800
CGCCTAAATA CAGCTGCATT CTATGATGCC GGGCTTTGGA CAAGAGCTCT       2850
TTCTCAGCTC CGTTTGTCCT CCCTCCCTTT TCCCCCTTCT TGCTAAATGC       2900
CTTTCTTTAC TTCTTTCTTC CCTTCCCTCC CCTATCGCAG CAGCCTCTCG       2950
GTGTAGGCTT TCCACGCTGC TGATCGGTAC CGCTCTGCCT CCTCTACGGG       3000
GTCTGAGGCC TTGAGGATGC CCCGGCCCAC AATGGCAATG TCGCTGCCGG       3050
CGATGCCAAT CAGCTTGTGC GGCGTGTTGT ACTGCTGGCC CTGGCCGTCT       3100
CCACCGACCG ATCCGTTGGT CTGCTGGTCC TCGTCTTCGG GGGGCAGCTG       3150
GCAGCCGGGC GTCATGTGGA TAAAGGCATC GTCGGGCTCG GTGTTGAGCG       3200
TCTCCTGCGA GATGAAGCCC ATGACAAAGT CCTTGTGCTC CCGGGCGGCC       3250
TCGACGCAGG CCTGCGTGTA CTCCTTGTTC ATGAAGTTGC CCTGGCTGGA       3300
CATTTGGGCG AGGATCAGGA GGCCTCGGCT CAGCGGCGCC TCCTCGATGC       3350
CCGGGAAGAG CGACTCGTCG CCCTCGGCGA TGGCCTTTGT TAACCGGGGC       3400
GAGGAGACGG ACTCGTACTG CTGGGTGACG GTGGTGATGG AGACGATGCT       3450
GCCCTTGCGG CCGTCGCCGG ACCGGTTCGA GTAGATGGGC TTGTCCAGGA       3500
CGCCAATGGA GCCCATGCCG TTGACGGCGC CGGCGGGCTC GGCGTCCCTG       3550
GAGTCGGCGT CGTCGTCAAA CGAGTCCATG GTGGGCGTGC CGACGGTGAC       3600
GGACGTCTTG ACCTCGCAGG GGTAGCGCTC GAGCCAGCGC TTGGCGCCCT       3650
GGGCCAGCGA GGCCACCGAC GCCTTGCCGG GCACCATGTT GACGTTGACA       3700
ATGTGCGCCC AGTCGATGAT GCGCGCCGAC CCGCCCGTGT ACTGCAGCTC       3750
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CGTGCTTGCG CGCCAGCGAC GCCAGCTGGG CTCCCGTGCC CGTCTCCGGG       3850
TGGAAGTCCC AGCCCGAGAC CATGTCGTAG TGCGTCTTGA GCACGACAAT       3900
CGACGGGCCA ATCTTGTCGG CCAGGTACAG CAGCTCGCGC GCTGTCGGCA       3950
CGTCGGCGCT CAGGCACAGG TTGGACGCCT TGAGGTCCAT GAGCTTGAAC       4000
AGGTAAGCCG TCAGCGGGTG CGTCGCCGTC TCGCTCCTGG CCGCGAAGGT       4050
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TTTAGAGGGG GGGAAAAAAA AGAGAGAGAT GGCACAATTC TGCTGTGCGA       4200
ATGACGTTGG AAGCGCGACA GCCGTGCGGG AGGAAGAGGA GTAGGAACTG       4250
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TGAATGGGGA ATGAGGAGCG CGATGGATTC GCTTGCATGT CCCCTGGCCA       4550
AGACGAGCCG CTTTGGCGGT TTGTGATTCG AAGGTGTGTC AGCGGAGGCG       4600
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ATCATAAGTA CGTATGTGCT TTTTCCTGCA AATGGTACGT ACGGACAGTT       4800
CATGTTGTCT GTCATCCCCC ACTCAGGCTC TCATGATCAT TTTATGGGAC       4850
TGGGGTTTTG CTGACTGAAT GGATTCAGCC GCACGAAACA AATTGGGGGC       4900
```

FIGURE 9C: pTEX2

```
CATGCAGAAG GGAAGCCCCC CCAGCCCCCT GTTCATAATT TGTTAAGAGT       4950
CGGAGAGCTG CCTAGTATGA AGCAGCAATT GATAACGTTG ACTTTGCGCA       5000
TGAGCTCTGA AGCCGGGCAT ATGTATCACG TTTCTGCCTA GAGCCGCACG       5050
GGACCCAAGA AGCTCTTGTC ATAAGGTATT TATGAGTGTT CAGCTGCCAA       5100
CGCTGGTTCT ACTTTGGCTC AACCGCATCC CATAAGCTGA ACTTTGGGAG       5150
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CCAAGTCTCA AACTGACTGC AGGCGGCCGC CATATGCATC CTAGGCCTAT       5700
TAATATTCCG GAGTATACGT AGCCGGCTAA CGTTAACAAC CGGTACCTCT       5750
AGAACTATAG CTAGCATGCG CAAATTTAAA GCGCTGATAT CGATCGCGCG       5800
CAGATCCATA TATAGGGCCC GGGTTATAAT TACCTCAGGT CGACGTCCCA       5850
TGGCCATTCG AATTCGTAAT CATGGTCATA GCTGTTTCCT GTGTGAAATT       5900
GTTATCCGCT CACAATTCCA CACAACATAC GAGCCGGAAG CATAAAGTGT       5950
AAAGCCTGGG GTGCCTAATG AGTGAGCTAA CTCACATTAA TTGCGTTGCG       6000
CTCACTGCCC GCTTTCCAGT CGGGAAACCT GTCGTGCCAG CTGCATTAAT       6050
GAATCGGCCA ACGCGCGGGG AGAGGCGGTT TGCGTATTGG GCGCTCTTCC       6100
GCTTCCTCGC TCACTGACTC GCTGCGCTCG GTCGTTCGGC TGCGGCGAGC       6150
GGTATCAGCT CACTCAAAGG CGGTAATACG GTTATCCACA GAATCAGGGG       6200
ATAACGCAGG AAAGAACATG TGAGCAAAAG GCCAGCAAAA GGCCAGGAAC       6250
CGTAAAAAGG CCGCGTTGCT GGCGTTTTTC CATAGGCTCC GCCCCCCTGA       6300
CGAGCATCAC AAAAATCGAC GCTCAAGTCA GAGGTGGCGA AACCCGACAG       6350
GACTATAAAG ATACCAGGCG TTTCCCCCTG GAAGCTCCCT CGTGCGCTCT       6400
CCTGTTCCGA CCCTGCCGCT TACCGGATAC CTGTCCGCCT TTCTCCCTTC       6450
GGGAAGCGTG GCGCTTTCTC ATAGCTCACG CTGTAGGTAT CTCAGTTCGG       6500
TGTAGGTCGT TCGCTCCAAG CTGGGCTGTG TGCACGAACC CCCCGTTCAG       6550
CCCGACCGCT GCGCCTTATC CGGTAACTAT CGTCTTGAGT CCAACCCGGT       6600
AAGACACGAC TTATCGCCAC TGGCAGCAGC CACTGGTAAC AGGATTAGCA       6650
GAGCGAGGTA TGTAGGCGGT GCTACAGAGT TCTTGAAGTG GTGGCCTAAC       6700
TACGGCTACA CTAGAAGAAC AGTATTTGGT ATCTGCGCTC TGCTGAAGCC       6750
AGTTACCTTC GGAAAAAGAG TTGGTAGCTC TTGATCCGGC AAACAAACCA       6800
CCGCTGGTAG CGGTGGTTTT TTTGTTTGCA AGCAGCAGAT TACGCGCAGA       6850
AAAAAAGGAT CTCAAGAAGA TCCTTTGATC TTTTCTACGG GGTCTGACGC       6900
TCAGTGGAAC GAAAACTCAC GTTAAGGGAT TTTGGTCATG AGATTATCAA       6950
AAAGGATCTT CACCTAGATC CTTTTAAATT AAAAATGAAG TTTTAAATCA       7000
ATCTAAAGTA TATATGAGTA AACTTGGTCT GACAGTTACC AATGCTTAAT       7050
CAGTGAGGCA CCTATCTCAG CGATCTGTCT ATTTCGTTCA TCCATAGTTG       7100
CCTGACTCCC CGTCGTGTAG ATAACTACGA TACGGGAGGG CTTACCATCT       7150
GGCCCCAGTG CTGCAATGAT ACCGCGAGAC CCACGCTCAC CGGCTCCAGA       7200
TTTATCAGCA ATAAACCAGC CAGCCGGAAG GGCCGAGCGC AGAAGTGGTC       7250
CTGCAACTTT ATCCGCCTCC ATCCAGTCTA TTAATTGTTG CCGGGAAGCT       7300
AGAGTAAGTA GTTCGCCAGT TAATAGTTTG CGCAACGTTG TTGCCATTGC       7350
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FIGURE 9D: pTEX2

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TACAGGCATC GTGGTGTCAC GCTCGTCGTT TGGTATGGCT TCATTCAGCT      7400
CCGGTTCCCA ACGATCAAGG CGAGTTACAT GATCCCCCAT GTTGTGCAAA      7450
AAAGCGGTTA GCTCCTTCGG TCCTCCGATC GTTGTCAGAA GTAAGTTGGC      7500
CGCAGTGTTA TCACTCATGG TTATGGCAGC ACTGCATAAT TCTCTTACTG      7550
TCATGCCATC CGTAAGATGC TTTTCTGTGA CTGGTGAGTA CTCAACCAAG      7600
TCATTCTGAG AATAGTGTAT GCGGCGACCG AGTTGCTCTT GCCCGGCGTC      7650
AATACGGGAT AATACCGCGC CACATAGCAG AACTTTAAAA GTGCTCATCA      7700
TTGGAAAACG TTCTTCGGGG CGAAAACTCT CAAGGATCTT ACCGCTGTTG      7750
AGATCCAGTT CGATGTAACC CACTCGTGCA CCCAACTGAT CTTCAGCATC      7800
TTTTACTTTC ACCAGCGTTT CTGGGTGAGC AAAAACAGGA AGGCAAAATG      7850
CCGCAAAAAA GGGAATAAGG GCGACACGGA AATGTTGAAT ACTCATACTC      7900
TTCCTTTTTC AATATTATTG AAGCATTTAT CAGGGTTATT GTCTCATGAG      7950
CGGATACATA TTTGAATGTA TTTAGAAAAA TAAACAAATA GGGGTTCCGC      8000
GCACATTTCC CCGAAAAGTG CCACCTGACG TCTAAGAAAC CATTATTATC      8050
ATGACATTAA CCTATAAAAA TAGGCGTATC ACGAGGCCCT TTCGTCTCGC      8100
GCGTTTCGGT GATGACGGTG AAAACCTCTG ACACATGCAG CTCCCGGAGA      8150
CGGTCACAGC TTGTCTGTAA GCGGATGCCG GGAGCAGACA AGCCCGTCAG      8200
GGCGCGTCAG CGGGTGTTGG CGGGTGTCGG GGCTGGCTTA ACTATGCGGC      8250
ATCAGAGCAG ATTGTACTGA GAGTGCACCA TAAAATTGTA AACGTTAATA      8300
TTTTGTTAAA ATTCGCGTTA AATTTTTGTT AAATCAGCTC ATTTTTTAAC      8350
CAATAGGCCG AAATCGGCAA AATCCCTTAT AAATCAAAAG AATAGCCCGA      8400
GATAGGGTTG AGTGTTGTTC CAGTTTGGAA CAAGAGTCCA CTATTAAAGA      8450
ACGTGGACTC CAACGTCAAA GGGCGAAAAA CCGTCTATCA GGGCGATGGC      8500
CCACTACGTG AACCATCACC CAAATCAAGT TTTTTGGGGT CGAGGTGCCG      8550
TAAAGCACTA AATCGGAACC CTAAAGGGAG CCCCCGATTT AGAGCTTGAC      8600
GGGGAAAGCC GGCGAACGTG GCGAGAAAGG AAGGGAAGAA AGCGAAAGGA      8650
GCGGGCGCTA GGGCGCTGGC AAGTGTAGCG GTCACGCTGC GCGTAACCAC      8700
CACACCCGCC GCGCTTAATG CGCCGCTACA GGGCGCGTAC TATGGTTGCT      8750
TTGACGTATG CGGTGTGAAA TACCGCACAG ATGCGTAAGG AGAAAATACC      8800
GCATCAGGCG CCATTCGCCA TTCAGGCTGC GCAACTGTTG GGAAGGGCGA      8850
TCGGTGCGGG CCTCTTCGCT ATTACGCCAG CTGGCGAAAG GGGGATGTGC      8900
TGCAAGGCGA TTAAGTTGGG TAACGCCAGG GTTTTCCCAG TCACGACGTT      8950
GTAAAACGAC GGCCAGTGCC                                       8970
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